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A recent report has confirmed our hypothesis that CYP1B1 is a gene that can regulate organ development. In the past year, we have established quantitation of the very low levels of CYP1B1 and CYP1A1 in cultured breast epithelia from 7 donors. Metabolism of 7,12-dimethylbenz(a)anthracene in these cells is initially due to CYP1B1 in 5 of 7 donors, but CYP1A1 contribute about half of the activity for 2 donors. Prolonged exposure to the DMBA or pre-exposure to the environmental toxicant dioxin elevated the total activity and contribution of the much more active CYP1A1. Turnover numbers and product selectivity for these forms for DMBA were comparable to what we observed for recombinant human CYP1B1 and CYP1A1. Equivalent analyses are being completed for estradiol. DNA adducts from dibenzo(al)pyrene and the 11,12 dihydrodiol were measured in HBEC from 3 donors. Dihydrodiol epoxide adducts to dA and dG were similar for each donor under conditions where complete metabolism had occurred. We have established that 2-ethynylphenanthrene (2.5 μM) and methyl-1-ethynyl pyrene (2.5 μM) are selective inhibitors of respectively CYP1B1 and CYP1A1. Cultured HB fibroblasts (HBF), but not the epithelia, expressed normal estrogen receptors. Growth of HBF was stimulated by estradiol (E_2) which may, therefore, mediate efforts of E_2 on epithelia. HBF expressed CYP1B1 but not CYP1A1 and differed substantially between donors in this respect, suggesting individual differences in regulation.

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Introduction

This proposal addresses potential mechanisms for contributions from environmental chemicals to the etiology of breast cancer. In particular, this research examines potential ways in which organochlorine compounds (OC's) and polycyclic aromatic hydrocarbons can synergize in producing biological effects in human breast cells. These ubiquitous environmental pollutants readily concentrate in the breast adipose tissue. PAH's generate DNA adducts that can potentially mediate mutagenesis and this process can be enhanced by the additional presence of OC's. Mutations in Ras and p53 genes that are consistent with PAH mutagenesis have been detected in human breast cancers. Epidemiology studies point to organochlorine compounds (OC's) that accumulate in breast adipose as a risk factor. These mechanisms may be synergistic. PAH's require activation to the ultimately carcinogenic form, the bay-region dihydrodiol epoxide, and this step involves P450 cytochromes. This laboratory has recently cloned a novel cytochrome P450, CYP1B1, that is particularly active in this process (1, 2). This form is related to a second P450 form, CYP1A1 that also metabolizes PAH (3). Each form is induced via the Ah-receptor by dibenzodioxins and planar PCB's, and this laboratory has provided evidence that these forms also metabolize 17β-estradiol to 2- and 4-catecholestrogens (4). CYP1B1 seems to be selectively effective in forming 4-catecholestrogens. This conversion has recently been shown to be elevated in endometrial and breast cancers. Estrogens also exert a potentiating effect on the stimulation of CYP1A1 by Ah-receptor ligands (5). Two additional mechanisms have been identified in our proposal; (a) estrogenic/anti-estrogenic activity of DDE, PCB metabolites (b) Ca^{++}-elevating effects of many OC's An exciting recent report (6) shows that several OC's synergize in their binding and activation of the estrogen receptor. A 100-fold increase in potency may emerge from this synergism. On the other hand, other work points to strong antiestrogenic effects of AhR ligands (7).

CYP1B1 is selectively expressed in hormonally regulated steroidogenic tissues (adrenal, ovary, testis), in the stromal cells of steroid sensitive tissues (mammary, prostate, uterus), and during limited periods in the embryo in tissue undergoing morphogenesis (1, 2). Interestingly, a dificiency in human CYP1B1 protein expression has recently been shown to be responsible for the development of congenital glaucoma (8). Thus, CYP1B1 may be a key determinant of the level of an agent that directly regulates tissue development, including cancer cells. We have hypothesized, based on the stromal expression pattern, that CYP1B1 may modulate stromal effects on epithelia, a key regulatory mechanism in the breast. Consequently, we are studying the expression of CYP1B1 in mammary stroma from different sources, including normal breast versus breast tumor. Ultimately, we will test whether CYP1B1 activity is related to stromal effects on mammary epithelia.

A key goal of our work has been to determine whether the estrogen receptor (ER) plays a selective role in transcriptional activations mediated by the Ah receptor. To this end, we have obtained human mammary cell lines that differ primarily through presence or absence of the ER. A critical problem here is that selection processes involved with the isolation of these lines are likely to introduce substantial adaptive secondary effects, notably the introduction of autocrine growth factors that take over the growth promoting role of ER. These selection effects are avoided here by comparison of ER^- cells with the ER^+ counterparts treated briefly with a potent ER antagonist ICI. This of course does not discriminate between direct nuclear effects of ER and secondary signalling effects of ER-enhanced factors.

Carcinogenic activity of polycyclic hydrocarbons in human mammary epithelial cells is almost certainly mediated by formation of DNA adducts. We have established a collaboration with Dr. William Baird's laboratory at Purdue University to measure DNA adducts formed in human breast epithelia by polycyclic aromatic hydrocarbons (PAH's) 7.12-dimethyl benzanthracene (DMBA) and dibenzo[al]pyrene (DB[al]P) and associated dihydrodiols. In these experiments activations from basal levels of CYP1B1 are being

compared with activations resulting from PAH-induction and from TCDD-induction. Adduct levels are being correlated with levels of CYP1B1 and CYP1A1 expression in the same cells.

PROGRESS IN YEAR 3:

This report summarizes work completed in accordance to the revised statement of work submitted in November, 1996.

AIM 1- Quantitation of CYP1B1 and CYP1A1:

In the previous report we described rtPCR of 19 breast tumor samples and 9 normal tissue samples. Several publictions have presented detection of CYP1B1 and CYP1A1 in tumors at this level. We have chosen to improve our rtPCR methodology for the analysis of CYP expression in human tissues to a fully quantitative level by incorporating the use of digoxigenin-labeled oligonucleotide probes with the generation of competitive PCR-mimic constructs. The former provides higher sensitivity and steeper standardization correlations which improve accuracy (Fig. 1). The latter utilizes an exogenous internal standard in competitive PCR, during which both the internal standard and the target cDNA compete for the same set of primers during the amplification reaction. The mimic constructs are currently being generated. The use of PCR mimics in this manner allows for the quantitation of the actual number of target DNA molecules added to the PCR reaction or, alternatively, relative changes in mRNA levels among the individual samples examined. We will complete a paper employing this technique within the next three months which will include the following:

1. CYP1B1 and CYP1A1 mRNA quantitative analysis of at least 19 tumor and 9 normal tissue samples. The analysis will include cDNA isolated from both normal and tumor tissues from four of the donors. We hope to obtain an additional 10 tumor samples from Midwestern division of the Cooperative Human Tissue Network.

2. Immunoblot analysis of Ah-receptor (AhR) and aryl hydrocarbon nuclear translocator (Arnt) protein expression in these individuals with respect to GAPDH and SP1 (cytosolic and nuclear internal standards, respectively). We have shown that while the expression of ARNT in these tumors is abundant, the AhR expression reflected wide variability between donors that did not reflect their tumor progression.

3. Comparison of CYP1B1, CYP1A1, AhR and Arnt expression, *in vivo* , with expression in two sets of established cells lines which present *in vitro* models for tumor progression:
   A. 21NT, 21PT, and 21MT-2, representative of increasing stages of tumor progression.
   B. ZR75.1, Hs578T, and MDA-MB-468 representing increasing metastatic potential.

4. Examination of CYP1B1 and CYP1A1 expression by *in situ* hybridization analysis of two of the donors (normal and tumor tissue), in collaboration with Dr. Judith Weisz (Hershey Medical Center, Hershey, PA).

This study will provide a comparative quantitative analysis of the expression of CYP1B1 and CYP1A1, *in vivo*, in normal- and tumor-derived tissues. These experiments

will facilitate the analysis of CYP1B1 and CYP1A1 expression with respect to the ER and tumorigenic status of the cell.

AIM 2- CYP1B1 in Human Breast cells:

A. Estradiol metabolism in cultured HMEC and tumor cell lines:

The analysis of estradiol (E_2) metabolism in cultured cultured HMEC is being completed in collaboration with Dr. Joachim Liehr (University of Texas Medical Center, Galveston, TX). Previous studies utilizing human uterine myometrium and the immortalized human mammary carcinoma, MCF-7, cell line have shown that CYP1B1 and CYP1A1 mediate the oxidative metabolism of E_2 generating 4-hydroxycatecholestrogen and the 2-hydroxycatecholestrogen, respectively (9). We have now prepared microsomes from cultured human mammary epithelial cells which have already been fully characterized for CYP expression and DMBA metabolism (Aim 2B). We have also prepared microsomes from various ER^+ and ER^- cell lines that have been similarly characterized:

A. T47D:A:18, ER^+; early stages of tumorigenesis
   T47D:C4:2W, ER^-
B. MDA-MB-231, ER^-; later stages of tumorigenesis
   MDA-MB-231/S30, ER^+.

These are currently being analyzed for estrogen 2- and 4-hydroxylation in comparison with microsomes which contain, exclusively, recombinant human CYP1B1 and CYP1A1. The analysis of the relationship between the CYP's, DMBA metabolism, and estrogen metabolism will form the material for one paper.

Our recent studies, utilizing cultured HMEC, have characterized CYP expression in heterogeneous cell populations consisting of at least two epithelial cell types, basal and luminal epithelia. Our results suggest that constitutive CYP1B1 and CYP1A1 are differentially expressed in the basal and luminal cell populations, respectively. Therefore, we feel it is extremely important to pursue the analysis of functional CYP expression (DMBA and estradiol metabolism analyses) in the isolated cell cultures. To date, we have completed the separation of basal and luminal cell populations (Fig. 2, 2A) from an organoid preparation of one individual (donor E), according to the method of Kao *et al.* (10). We are currently completing the separation procedures on organoid preparation from two additional donors (donor D and donor F), thus, representing individuals recently shown to express lower, intermediate, and higher levels of constitutive CYP1B1 and CYP1A1 microsomal protein:

A. Donor D; CYP1A1, ≤0.02-pmol/mg; CYP1B1, 0.6-pmol/mg.
B. Donor E; CYP1A1, 0.05-pmol/mg; CYP1B1, 0.7-pmol/mg.
C. Donor F; CYP1A1, 0.16-pmol/mg; CYP1B1, 1.2-pmol/mg.

This will provide an analysis of functional CYP1B1 and CYP1A1 expression in distinct basal and luminal cell populations in which CYP expression has already been characterized in the heterogeneous culture. We will complete a manuscript in the next several months which will include the following:

1. Characterization of CYP1B1 and CYP1A1 expression in separated basal and luminal cell populations by immunoblot analysis.

2. Immunocytochemical analysis of CYP1B1 and CYP1A1 expression in separated basal (expression of cytokeratin 14) and luminal (expression of cytokeratin 18) epithelial cell populations.

3. Analysis of DMBA metabolism (a marker of functional CYP activity) in the isolated cell populations.

4. Eludication of CYP1B1- versus CYP1A1-mediated E_2 metabolism using recombinant human CYP1B1 (rhCYP1B1) and recombinant hCYP1A1 (rhCYP1A1), each independently expressed in V79 cells.

5. Analysis of E_2 metabolism in separated basal and luminal epithelial cell populations of cultured HMEC of three donors

We predict that the level of 4- and 2-hydroxylation of E_2 will strongly correlate with the relative contributions of CYP1B1 and CYP1A1, respectively, to DMBA metabolism and CYP expression in donors D, E, and F as well as in the tumor cell lines.

B. Expression of P450 cytochromes in breast epithelial cells:

We have now completed two papers which provide a detailed analysis of the cytochrome P450 expression in cultured mammary epithelial cells (see attached appendix). One paper characterizes CYP1A1 and CYP1B1 expression in cultured HMEC from eight donors, while the other examines the effect of ER status on CYP1A1 and CYP1B1 expression using the T47D (A:18, ER^+; C4:2W, ER^-) and MDA-MB-231(ER^-; S30, ER^+) mammary tumor cell lines. During the past year, we have completed quantitation of the exact levels of CYP expression in the cultured HMEC and tumor cell lines, calculated their functionality by converting DMBA metabolism data to turnover numbers for each isoform, and compared these values with the turnovers we have calculated for recombinant human CYP1B1 and CYP1A1 standards. The result has been a remarkable concordance of data.

The analysis of the recombinant proteins has shown that CYP1B1 is 6-times less active than CYP1A1 in the metabolism of DMBA, a marker of functional CYP activity (Table 1). Thus, the cultured HMEC indicate a CYP1B1 predominance in mediating basal metabolism in cells from some donors, while others express sufficient CYP1A1, although at very low levels, to contribute substantially to basal activities. These donors demonstrating measurable levels of basal CYP1A1 activity would be expected to show an enhanced capability for the bioactivation of PAH's. TCDD-induced DMBA metabolism exceeded basal levels by >100-fold, but could be interpreted with similar turnover numbers for CYP1A1 and CYP1B1, whereby CYP1A1 contributes 75- to 85-percent of the activity and CYP1B1 contributes 15- to 25-percent of the activity (Table 2). The magnitude of CYP1B1 and CYP1A1 expression and associated activities are extremely low in these cultured cells, so this additional level of quantitation greatly solidifies the data. We have noted substantial variability in CYP expression among the donors, although this is greater for CYP1A1 (as much as 21-fold) than for CYP1B1 (as much as 7-fold) (Table 3). Quantitation of AhR and Arnt protein expression, proteins which mediate CYP expression, indicate little inter-individual variation (Fig. 3).

In the human mammary tumor cell lines, we have established that basal CYP1B1 is elevated in ER- cells, while induction of CYP1A1 is suppressed when compared to ER+ cells (Fig. 4, 5). Quantitation of specific content of CYP1A1 and CYP1B1 demonstrates similar levels of induced CYPs in ER+ and ER- T47D cells, as is observed in the primary HMEC (Table 4). Differences in expression between ER+ and ER- clones are within the range of inter donor variation for HMEC. The variations in CYP expression between ER+ and ER- clones do not arise directly for ER activity since the ER antagonist ICI 182,780 failed to convert CYP expression patterns in ER+ clones to ones similar to those observed in ER- clones (Fig. 6, Table 5). The ER+ and ER- MDA-MB-231 derived lines demonstrated the same trends in CYP expression as was observed in the T47D cells, however, levels of expression were much lower. Evidently, regulatory factors associated with the cell type have a dominant influence on CYP expression, compared to that of the ER alone. Notably, MDA-MB-231 cells (ER-) exhibited almost exclusive CYP1B1

expression, which is remarkably similar to what we have observed in human mammary fibroblasts. This is of interest because MDA-MB-231 cells have a fibroblast-like morphology and fibroblastic markers (11, 12).

In the past year we have collaborated with Dr. W. Baird (Purdue University, West Layfayette, IN) to examine formation of DNA adducts from dibenzo[al]pyrene (DB[al]P) and (±)-trans-dibenzo[al]pyrene-11,12-diol (DB[al]P-diol). Adducts were only observed following TCDD-induction (Table 6). Multiple dihydrodiol epoxide adducts with dA and dG were detected in nuclear extracts isolated from cells treated with both DB[al]P and DB[al]P-dihydrodiol,. However, the level of adduct formation was higher in cells treated with DB[al]P-dihydrodiol. The comparison of adduct formation in three donors that have been characterized for CYP1B1 and CYP1A1 expression demonstrated equivalent levels of adduct formation. The reason for this similarity of adduct levels, even when CYP levels varied by several fold, is that dB[al]P and the associated dihydrodiol are fully metabolized with the result that CYP's are not rate limiting. We are currently repeating this experiment with a 2 h cell incubation instead of 24 h to limit the metabolism We will assemble this data as a publication with Dr. Baird.

In order to relate this work to a carcinogenesis model and to *in vivo* regulation, we have analyzed CYP1B1 and CYP1A1 expression in the rat mammary gland. Experiments have been completed *in vivo* using two strains of inbred rats that display different susceptibilities to chemically-induced mammary cancer; Wistar/Furth (WF) being highly susceptible and Wistar/Kyoto (WK) being highly resistant. We have observed large differences in cytochrome P450 expression in the mammary gland following induction with ß-naphthoflavone, an agonist of the Ah receptor, with WF animals exhibiting 5-fold higher levels of induced CYP1B1 compared to WK. Conversely, WK animals displayed 3-fold higher levels of induced CYP1A1 in their mammary glands. In culture, the low level of CYP1B1 expression in rat mammary epithelial cells is enhanced 3-fold when the cells are grown on reconstituted basement membrane (matrigel). This extracellular matrix surface promotes functional differentiation of mammary epithelial cells, stimulating ductal morphogenesis and elevating cytochrome P450 expression to levels comparable to that seen *in vivo* (Fig. 7, 8).

We have observed analogous morphogenesis of luminal HMEC cultured on matrigel, and we anticipate similar increases in epression of CYP1B1 in human luminal epithelial cells cultured in the presence of matrigel. Examination of the effect of this *in vitro* morphogenesis on AhR activity and CYP expression in HMEC will be an important activity in the final year of this grant. This will be carried out in conjunction with sepation of the luminal and basal epithelial cell types.

AIM 3A- Impact of CYP-mediated estradiol metabolism on human breast cells:

We have used V79 cells which exclusively express either human CYP1A1 or CYP1B1 to identify selective inhibitors of functional activity. We have analyzed a series of ethynyl substituted PAH's which undergo metabolic activation to highly inhibitory ketenes (Table 7). Of seven inhibitors, methyl-1-ethynylpyrene (M1EP) selectively inhibited CYP1A1 activity (2.5 μM, 91-percent inhibiton of CYP1A1 versus 81-percent inhibition of CYP1B1). 2-Ethynylphenanthrene (2EPH) selectively inhibits CYP1B1 (2.5 μM, 34-percent inhibition of CYP1B1 versus 70-percent stimulation of CYP1A1 which we have shown in fibroblasts is the result of protein stabilization). These inhibitors are currently being in the analysis of isolated basal and luminal cultured HMEC to characterize the involvement of the respective CYP's in DMBA and estradiol metabolism in the intact cell. We will use the V79 cells expressing CYP1A1 or CYP1B1 as reference cultures. We expect to be able to obtain 60- to 80-percent inhibition of CYP1B1 (2EPH) with less than 10 percent inhibition of CYP1A1 following a 1-2 h pretreatment with the inhibitor. The reverse inhibition should be attained with M1EP. Establishing this selective inhibition

should provide a valuable tool for other investigators and will be sufficient for a publication.

AIM 3B:Activity in human mammary fibroblasts (HMF's):

Human mammary fibroblasts (HMF's) (tumors and peripheral) and human skin fibroblasts have been characterized with respect to CYP expression, DMBA metabolism, and levels of AhR and ARNT (Fig. 9, 10). We have examined cells from several donors. Although we had expected large differences related to the site of origin, we found substantial inter-donor differences which were more prominent than inter-site differences in cells from a single donor. For all HMF's, CYP1B1 was expressed constitutively and was induced by TCDD, while CYP1A1 was only marginally detected. Levels of CYP1B1 and DMBA metabolism were lower than in breast epithelial cells. AhR and ARNT levels were also much lower than in the epithelial cells. The level of constitutive and TCDD-induced CYP1B1 varied between donors. DMBA metabolite profiles of HMF metabolism, even when TCDD-induced, were typical of that of uninduced human mammary epithelia.

We have identified low levels of normal estrogen receptors in HMF's. Interestingly, the growth of breast fibroblasts from both normal tissues and tumors was stimulated by estradiol and inhibited by ICI 182,780 (estrogen receptor antagonist), while skin fibroblasts were unresponsive even though exhibiting similar receptor levels (Fig. 11). This finding for fibroblasts contrasts with that for epithelia where we detected a 58 kDa splice-variant estrogen receptor which lacks the DNA-binding domain. We are currently further characterizing this growth regulation by estradiol in terms of cell cycle.

Microsomes have been prepared for characterization of estrogen hydroxylation by constitutive and TCDD-induced fibroblasts, we expect predominance of 4-hydroxylation. These analyses will shortly be carried out along with those from epithelial cells (Aim 2).

Thus the first characterization of the regulation specific P450s in human breast fibroblasts together with estrogen regulation will be submitted to Cancer Research in the next 2 months.

AIM 4: Effects of estrogens and xeno-estrogen's on secretion from human mammary fibroblasts:

Following the identification of ER and estrogen responsiveness in human breast fibroblasts we have decided to combine and re-focus Aims 4 and 5 of the original proposal, so that the remaining work is included in Aims 3 and a new Aim 4. We now aim to :

a) Identify estrogen stimulation of secretion of growth factors from HMF's. Since ER may be activated independent of E_2, inhibition of ER by the antagonist ICI 182,780 will be used to indicate involvement of the receptor. This compound destabilizes the ER leading to much lower levels.

b) Find conditions where HMF's will enhance growth or morphology changes in human mammary epithelia. A recent poster at the Endocrine Society meeting reported E_2-stimulation of epithelial growth in the presence of HMF's.

At present we have identified secretion of HGF from HMF's while the receptor (c-Met) is localized predominantly in epithelial cells (we can detect c-Met in cultures of luminal and basal epithelia). Other proteins of interest include TGFα, TGFβ, fibronectin, IGF1, IGF2, and various interleukins. Many of these growth factors and cytokines are also sensitive to TCDD. We have seen that TCDD increases secretion of HGF from HMF's. We will use total ^{35}S-labeling coupled to 2D-gel electrophoresis to provide a general picture of E_2 effects on protein secretion and test specific antibodies for the above proteins in immunoblots. We will also initiate experiments to determine the impact of these secreted factors on breast epithelial cells in culture and how E_2 and TCDD change this secretion process.

When estrogen stimulation of HMF's has been characterized, we will determine whether selected environmental estrogens (xenoestrogens), such as DDT, 3,3',4,4'-tetrachlorobiphenyl, chlordecone, and dieldrin, will also produce stimulation of such secretion.

Conclusions

1. We have demonstrated that extremely low levels of CYP1A1 in human breast epithelial cells contribute significantly to bioactivation of polycyclic hydrocarbons. Specific activities of CYP1A1 and CYP1B1 in these cells, based on expression levels, are comparable to recombinant forms. Expression of these forms and, particularly CYP1A1, was highly variable between individual donors.

2. Human breast epithelial cells form dihydrodiol epoxide adducts with low amounts of dibenzo(al)pyrene (0.3μM). Levels do not differ between individual donors under conditions where metabolism is complete.

3. Human breast fibroblasts exhibit detectable levels of estrogen receptors and show a positive growth response to estradiol. Normal estrogen receptors were absent from cultured epithelial cells. This supports the hypothesis that estrogens will stimulate epithelial changes via factors released from fibroblasts.

4. Human breast fibroblasts express P4501B1 but not P4501A1. Expression levels were also highly variable between donors, although much less variable between different sites (tumor, normal breast, skin) from the same donor.

In general, the progress in this research continues to support the concept that CYP1B1 and CYP1A1 may be important contributors to breast cancer that differ substantially between individuals. These cytochromes may participate through mediating physiological signalling or by activating environmental chemicals, like polycyclic hydrocarbons, which additionally induce their expression.

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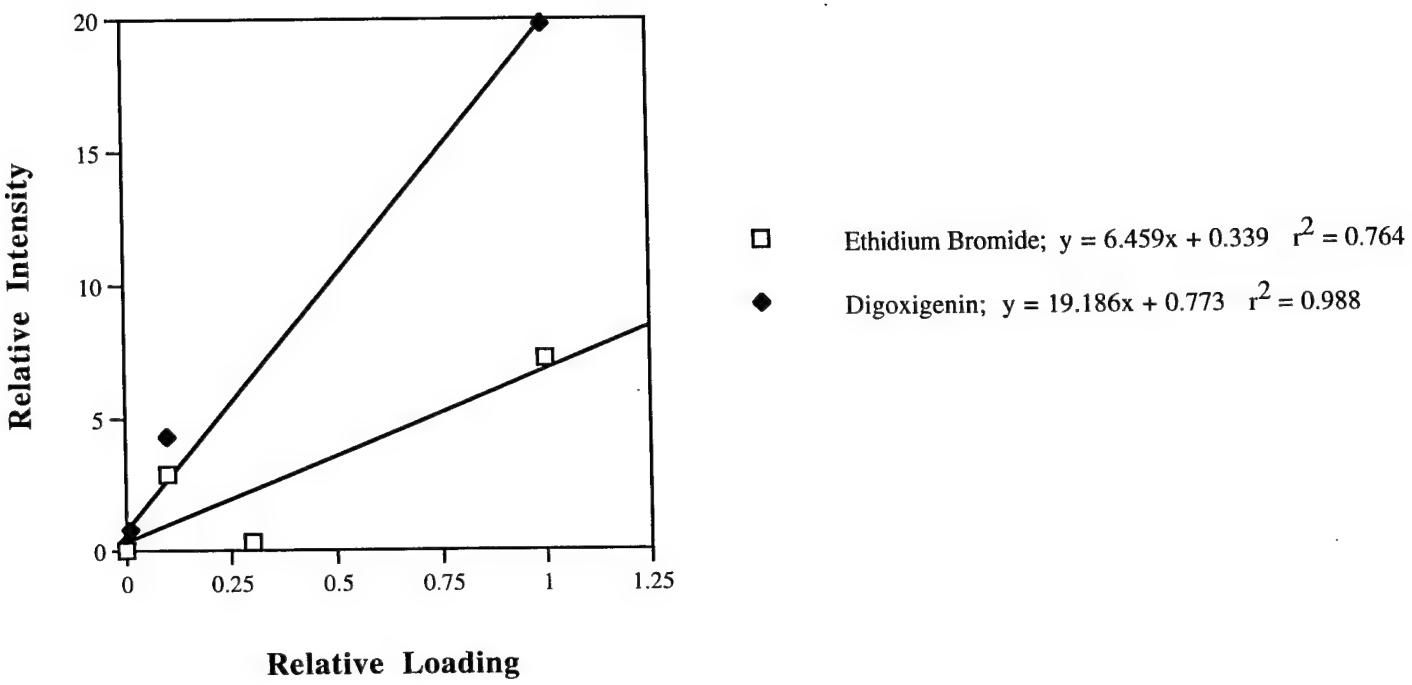
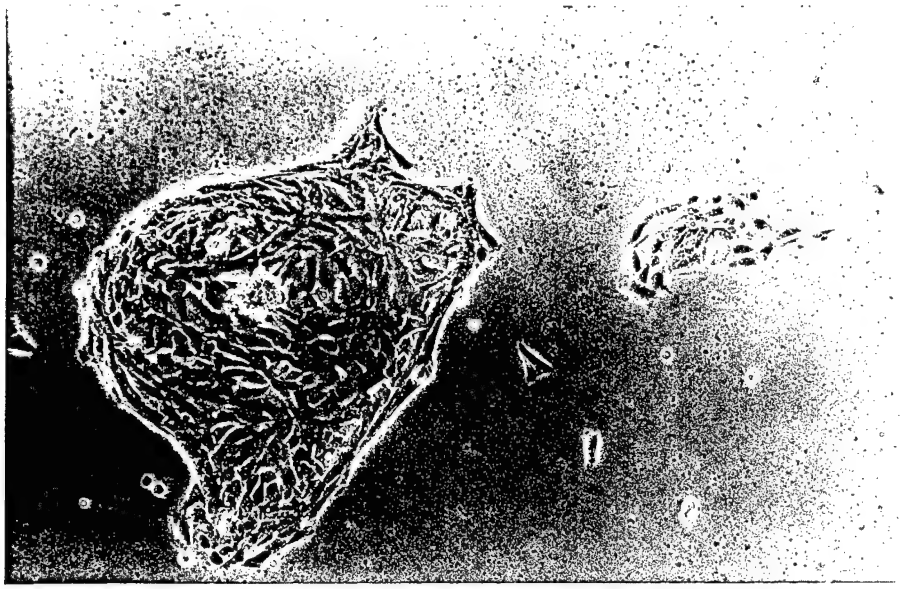


Figure 1. Quantitative standardization of rtPCR reaction products by digoxigenin and ethidium bromide detection methodologies. rtPCR reaction products were analyzed by slot blot analysis utilizing digoxigenin-labeled oligonucleotide cDNA probes. Ethidium bromide detection was completed utilizing 1.6% agarose gel chromatography. Relative quantitation of the standard curve was completed by soft laser scanning densitometry,

A.



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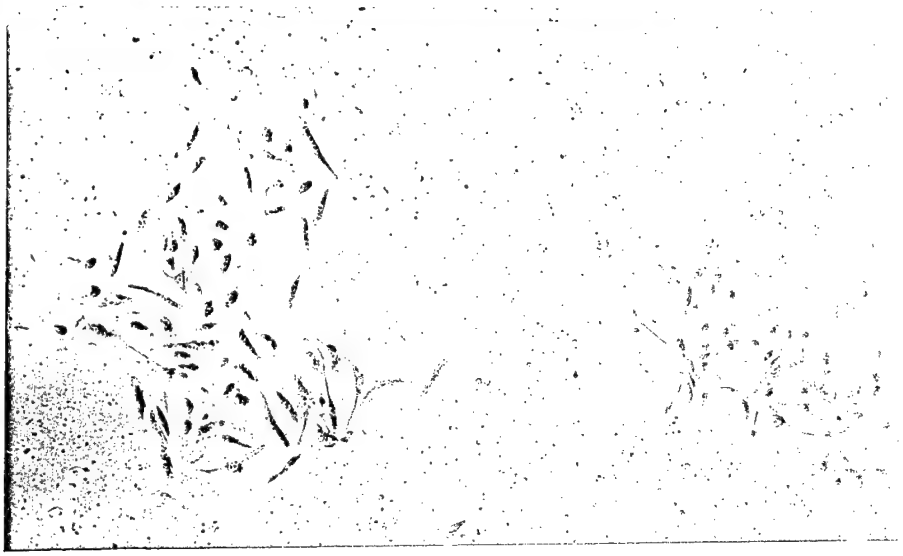

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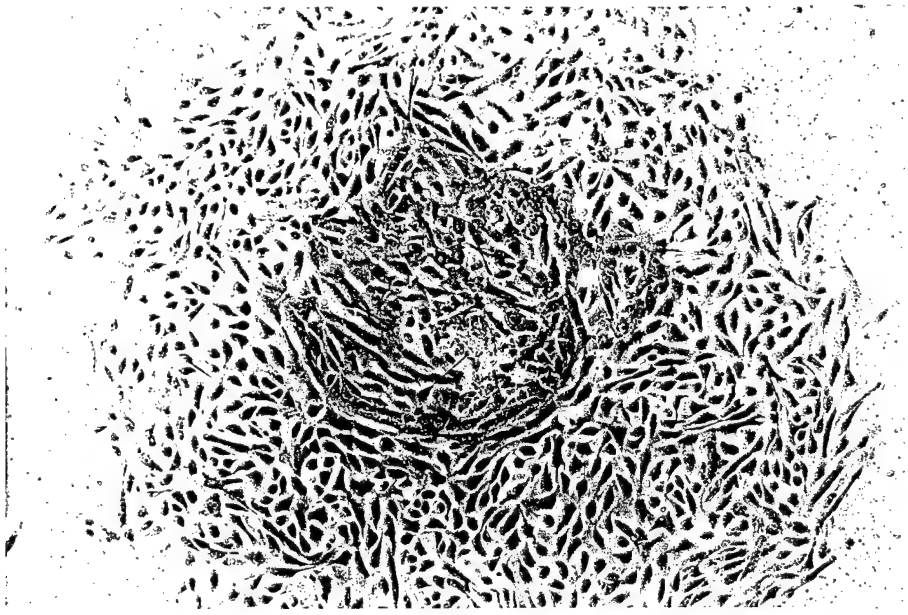


Figure 2. Primary HMEC organoid preparations cultured in MEGM media exhibiting regions of luminal (A), basal (B), and mixed luminal and basal epithelial cell proliferation.

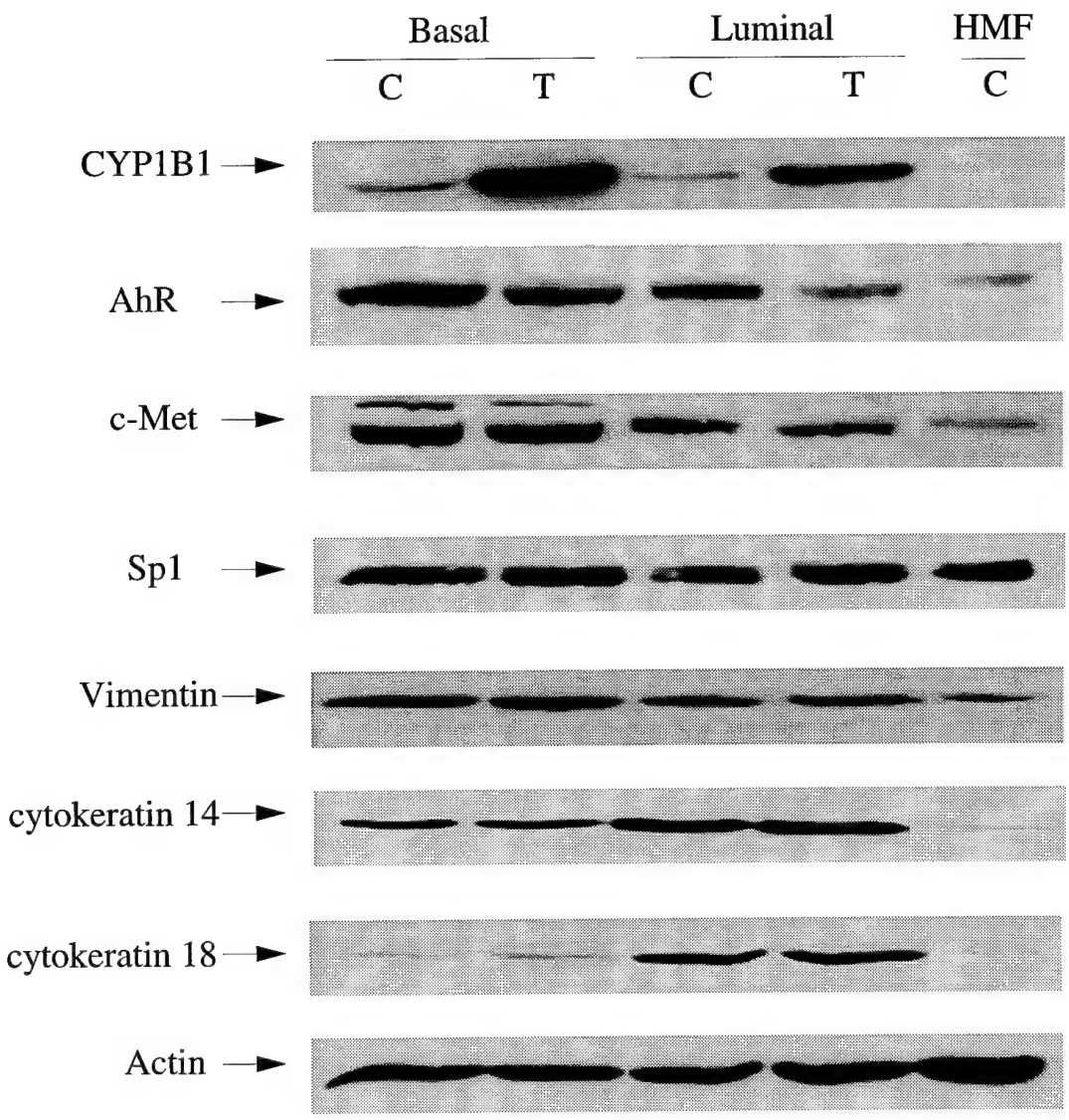


Figure 2A. Differential Expression of CYP1B1 and its regulator AhR, c-Met (HGF receptor), vimentin, cytokeratin 14 and 18 in luminal and basal separated primary human mammary epithelial cell culture in comparison to primary human mammary fibroblasts (HMF). Total cellular proteins were isolated from Trizole® lysates of these cells after they were treated with 10 nM TCDD for 24 h. Proteins were loadedans separated on SDS-gels , and proteins on the gels were transfered to a nitrocellulose membrane and probed with respective antibodies consecutively. The secondary antibodies were conjugated with horseraddish peroxidase, and specific bands were visualized with enhancedchemiluminescence (ECL) reagents kit. Extracts from untreated HMF was included for comaprison. Sp1 was probed for normalization of total nuclear proteins exraction recovery, and membranes were probed for b-actin protein as control for loading.
The data show the clear separation of the basal and luminal epithelia of the breast; where the cytokeratin 18 discriminately expressed in the luminal epithelia and CYP1A1 is similarly expressed in luminal epithelia, while CYP1B1 is preferentially expressed in basal epithelia which is more stromally localized.

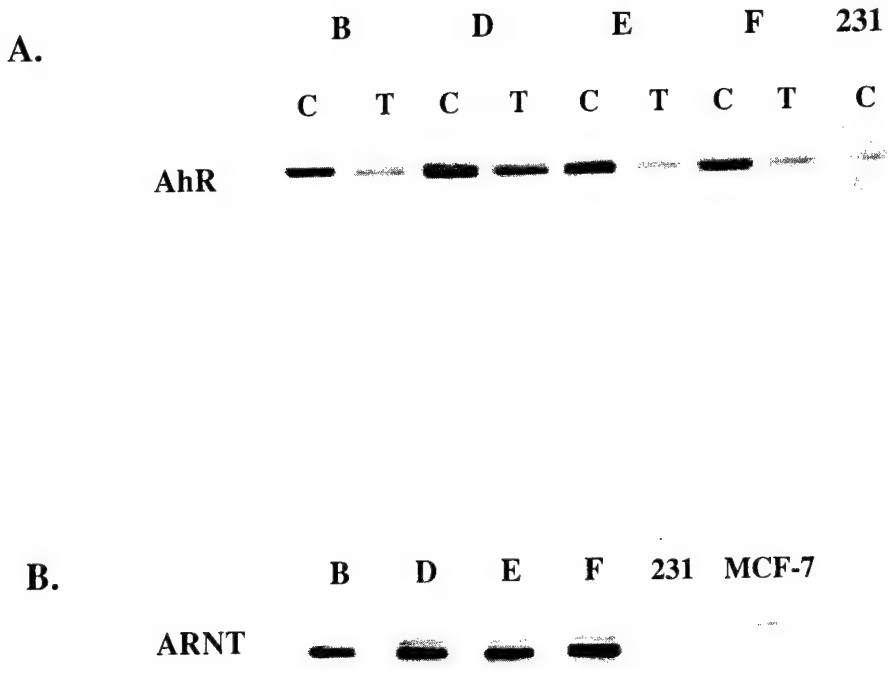


Figure 3. Immunoblot analysis of AhR (A, constitutive (C) and TCDD-induced (T), 10 nM for 24 h) and Arnt (B, constitutive expression in day 6 secondary HMEC cultures of four individual donors. Total proteins were isolated by TRIzol procedures and were analyzed by SDS-PAGE. Immunoreactive proteins were visualized by the ECL method of detection. MDA-MB-231 and MCF-7 protein fractions were utilized as reference standards for comparison with expression levels in immortalized human mammary cell lines. Protein loadings were 20μg/lane and membranes were exposed for 5 min.

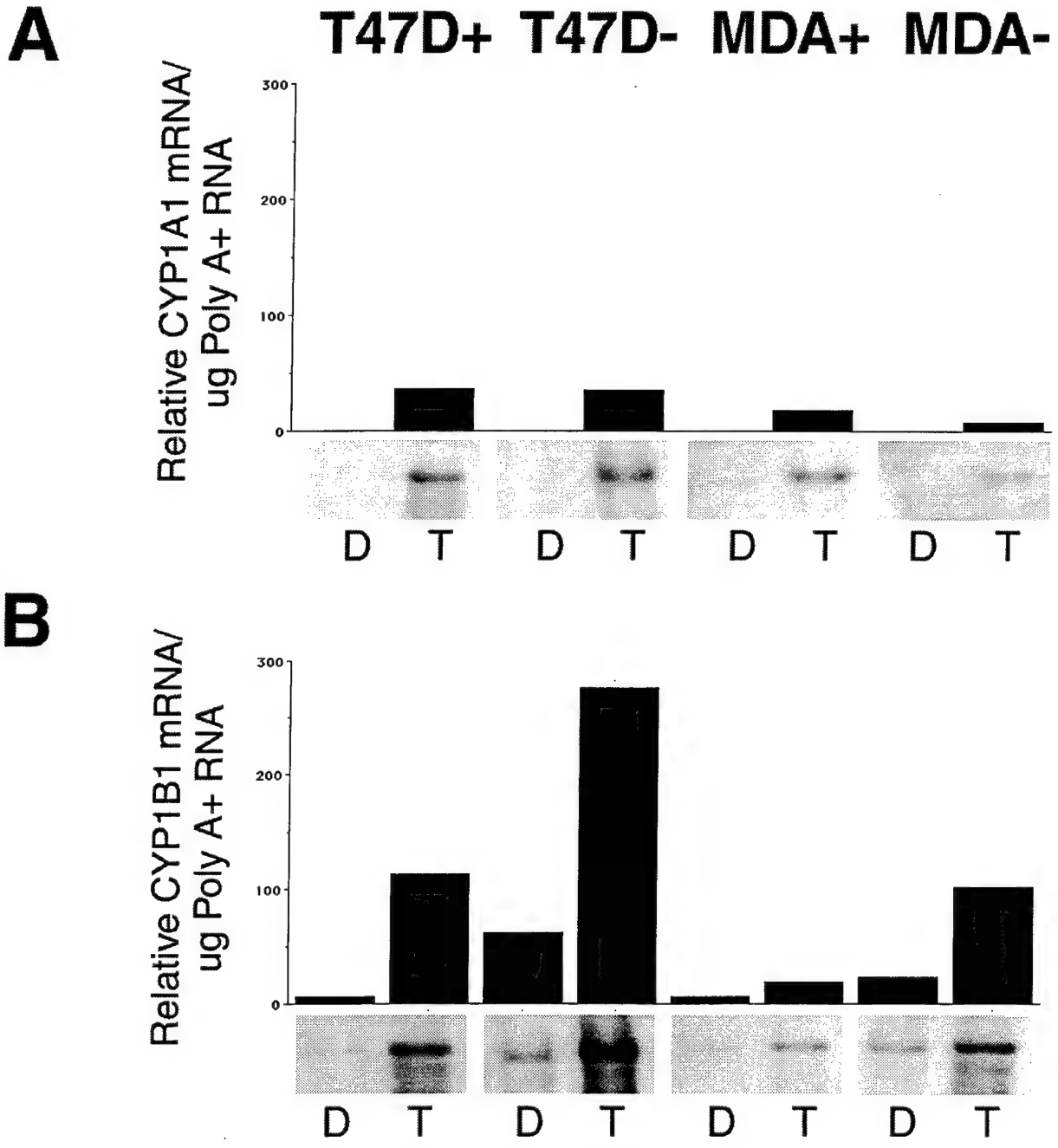


Figure 4. Relative CYP1A1 and CYP1B1 mRNA Levels in T47D and MDA cells treated with or without TCDD. D=DMSO treated, T=TCDD treated

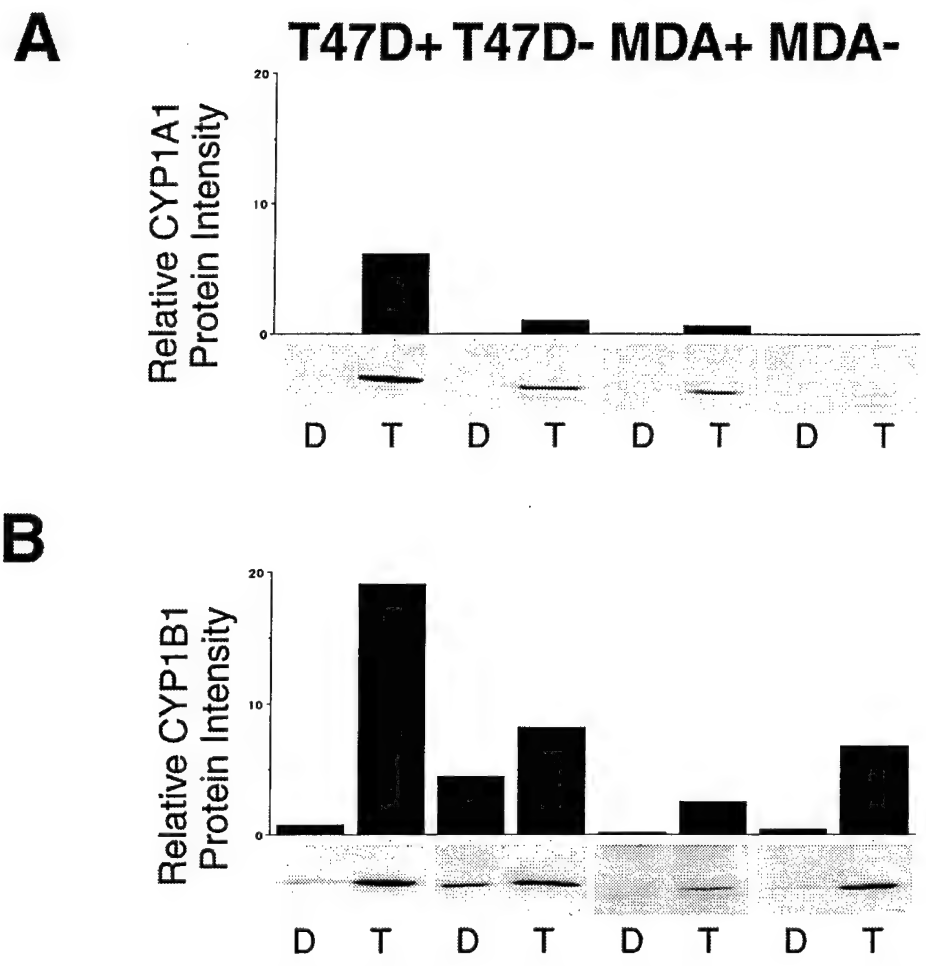


Figure 5. Determination of relative CYP1A1 and CYP1B1 protein levels in T47D and MDA cells treated with or without TCDD. D=DMSO treated, T=TCDD treated. Ordinate measured in undefined units.

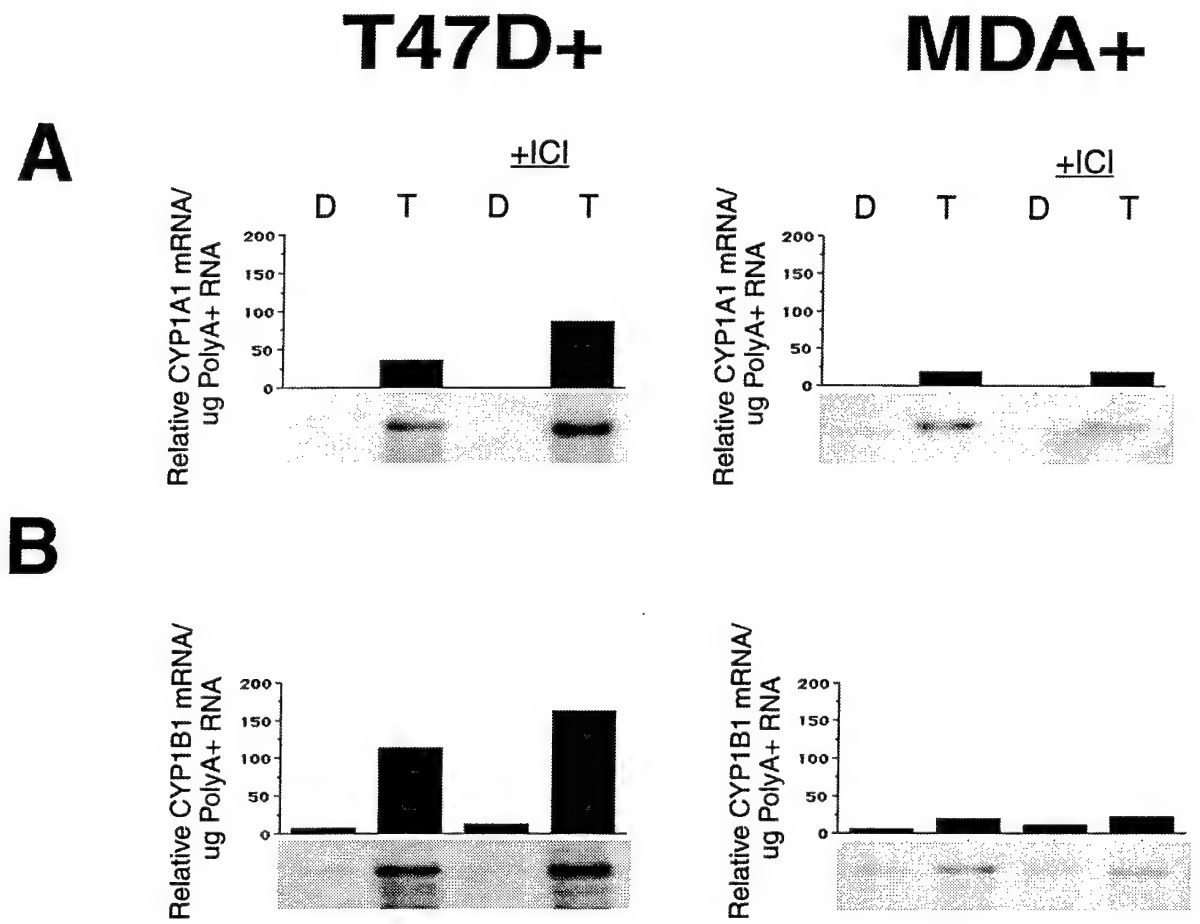


Figure 6. Effects of ICI 182,780 On CYP1A1 and CYP1B1 Expression In T47D and MDA Cells. D=DMSO treated, T=TCDD treated

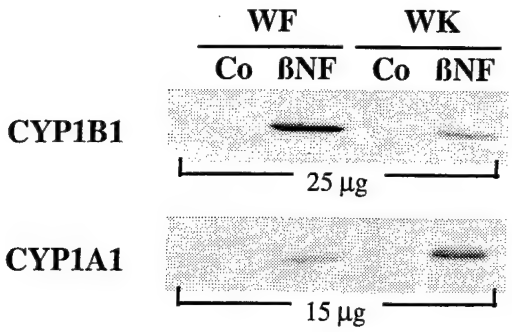


Figure 7. **Comparison of CYP1B1 and CYP1A1 expression in Wistar/Furth and Wistar/Kyoto rats following induction with ß-naphthoflavone.** Female Wistar/Furth (WF) and Wistar/Kyoto (WK) rats (virgin, 8-9 weeks old) were treated with vehicle control (Co) or ß-naphthoflavone (ßNF) (60 mg/kg, i.p., 3 consecutive days). Twenty-four hours after the last injection mammary tissue was collected and microsomal protein isolated. Microsomal protein (loadings indicated below figures) was separated by SDS-PAGE, transferred to nitrocellulose membranes, probed for CYP1B1 and CYP1A1 immunodetectable protein using antibodies specific to each form, and visualized by the enhanced chemiluminescence method.

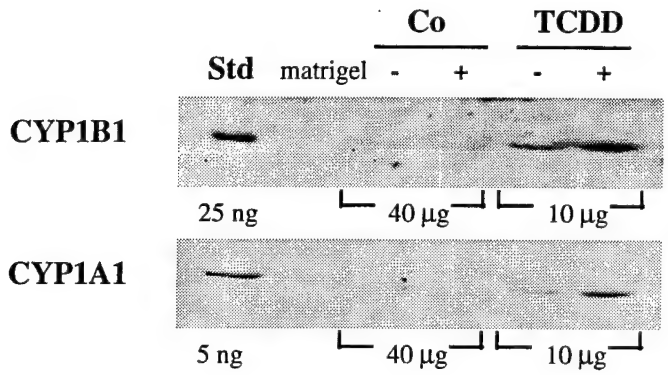


Figure 8. **Effect of matrigel on CYP1B1 and CYP1A1 expression in rat mammary epithelial cells in response to TCDD.** Rat mammary epithelial cells were isolated from female Sprague-Dawley rats (virgin, 55 days old) and cultured on plastic (-) or matrigel (+) as indicated for 1 day. Cells were then treated with 0.1% DMSO (Co) or 10^{-9}M TCDD for 24 h, the cells collected and microsomal protein isolated. Microsomal protein (loadings indicated below figures) was separated by SDS-PAGE, transferred to nitrocellulose membranes, probed for CYP1B1 and CYP1A1 immunodetectable protein using antibodies specific to each form, and visualized by the enhanced chemiluminescence method. Purified CYP1B1 and CYP1A1 standards (Std) are added for controls, loadings as indicated.

Table 1. Microsomal DMBA Metabolism in Constitutive and TCDD-Induced Cultured HMEC

SAMPLE	Dihydrodiols 5,6-	8,9-	10,11-	3,4-	7-OHMMBA	Phenols A	B	Total DMBA Metabolism (pmol/mg/hr)
CONSTITUTIVE								
Donor D	0.3	0.6[a]		0.2	0	1.6	0.6	**3.3**
	(9)[b]	(18)		(6)		(48)	(18)	
Donor E	1.1	2.4	0.4	0.4	0	4.2	0.6	**9.1**
	(12)	(27)	(4)	(4)		(46)	(7)	
TCDD-INDUCED[e]								
Donor D	26	147	7	9	35	67	18	**309**
	(8)	(48)	(2)	(3)	(11)	(22)	(6)	
Recombinant Human Cytochromes P450								
CYP1B1[c]	50	33	27	17	4	154	2	**287**
	(17)	(12)	(10)	(6)	(1)	(54)	(<1)	
CYP1A1[d]	189	870	73	80	10	450	45	**1717**
	(11)	(51)	(4)	(5)	(<1)	(26)	(3)	

[a] DMBA 8,9 + 10,11-dihydrodiol, peaks were poorly resolved at this low level of activity.

[b] Percent metabolite distribution.

[c] Recombinant human CYP1B1 expressed in lymphoblast microsomes (Gentest Corp.), 74-pmol/mg protein.

[d] Recombinant human CYP1A1 expressed in lymphoblast microsomes (Gentest Corp.), 104-pmol/mg protein.

[e] 10nM TCDD for 24 hours prior to analysis.

Table 2. CYP1B1- and CYP1A1-Mediated DMBA Metabolism and Turnover in Cultured HMEC.

Donor	DMBA Metabolism (pmol/mg/h)	Turnover (pmol/mg)	
		CYP1B1	CYP1A1
HMEC			
Constitutive[a]	9	6	90
TCDD-Induced[b]	308	4	21
Recombinant Microsomes			
hCYP1B1[c]	287	4	
hCYP1A1[d]	1717		17

[a]Activity demonstrated by donor E.
[b]Activity demonstrated by donor D; 10 nM TCDD for 24 h prior to analysis.
[c]Human recombinant lymphoblast microsomes expressing CYP1B1 (74-pmol/mg; Gentest Corp).
[d]Human recombinant lymphoblast microsomes expressing CYP1A1 (104-pmol/mg; Gentest Corp).

Table 3. CYP1B1 and CYP1A1 Expression in Cultured HMEC.

Donor	CYP1B1[a] (pmol/mg)		CYP1A1[b] (pmol/mg)	
	Constitutive	TCDD[c]	Constitutive	TCDD
A	<0.01	2.3	<0.01	0.8
B	1.4	12.2	0.02	16.5
C	1.3	6.5	0.02	2.7
D	0.6	10.7	0.02	12.8
E	0.7	16.6	0.05	14.2
F	1.2	10.9	0.16	15.9
G	0.8	13.5	0.03	7.2

[a] Quantitated relative to recombinant human CYP1B1 standard curve (74 pmol/mg; lymphoblast microsomes, Gentest Corp.).
[b]Quantitated relative to recombinant human CYP1A1 standard curve (104 pmol/mg; lymphoblast microsomes, Gentest Corp.).
[c]10 nM TCDD for 24 h prior to analysis.

Table 4. Specific Content and Turnover of CYP1A1 and CYP1B1 in T47D and MDA Cells

	CYP1A1 Specific Content (pmol/mg cellular protein)	CYP1B1 Specific Content (pmol/mg cellular protein)
T47D+		
DMSO	n.d.	1.9
TCDD	15.1	35.6
T47D-		
DMSO	n.d.	8.6
TCDD	2.3	15.5
MDA+		
DMSO	n.d.	0.7
TCDD	3.6	5.0
MDA-		
DMSO	n.d.	1.2
TCDD	0.06	12.9

The pmoles of CYP1A1 and CYP1B1 for determination of specific content were determined by regression analysis against a standard curve of recombinant protein values and divided by the mg protein in the sample.

Table 5. Comparison of Treatments With/Without ICI 182,780

		±TCDD
	+ICI/-ICI	+ICI/-ICI
T47D±		
CYP1A1		
mRNA[a]	nd	2.9
Protein	nd	1.1
CYP1B1		
mRNA[a]	1.4	1.7
Protein	0.8	1.1
MDA±		
CYP1A1		
mRNA[a]	nd	1.4
Protein	nd	0.8
CYP1B1		
mRNA[a]	2.2	1.5
Protein	0.3	1.8

[a] Normalized to ß-actin content.

nd Not Detected

Table 6. Analysis of DNA-Adduct Formation in Cultured HMEC.

Donor	A		B		D	
	P[b]	-Diol[c]	P	-Diol	P	-Diol
Adduct						
anti-dG (1)	ND	1.4[a]	ND	1.5	ND	1.3
anti-dG (2)	1.2	2.6	1.0	3.4	1.6	3.4
anti-dG (3)	1.3	2.6	1.1	3.1	1.5	3.0
+syn-dA	4.2	ND	2.0	ND	3.4	ND
-anti-dA	2.4	8.0	2.1	10.1	3.5	9.2
+syn-dA (2)	2.5	ND	1.4	ND	2.5	ND

[a] Relative level of adduct formation as measured by ^{32}P-DNA postlabeling/HPLC methodology.
[b] Dibenzo[al]pyrene, P.
[c] (±)-trans-dibenzo[al]pyrene-11,12-diol.

Table 7. Analysis of the Inhibition of CYP1B1 and CYP1A1 Functional Activity by Selective PAH Inhibitors.

INHIBITOR	% REMAINING ACTIVITY[a]	
	CYP1B1[b]	CYP1A1[c]
Methyl-1-ethynylpyrene (M1EP)	9	19
1-Ethynylphrene (1EP)	57	35
Ethylpyrene (ETP)	185	170
9-Ethynylphenanthrene (9EPH)	88	98
2-Ethynylphenanthrene (2EPH)	170	66
Phenanthrene	141	111
1-(1-Propynyl)pyrene (1PP)	6	25

[a] All inhibitors (2 μM) were preincubated with cells for two hours prior to DMBA metabolism analysis.
[b] Recombinant human CYP1B1 expressed in V79 cells.
[c] Recombinant human CYP1A1 expressed in V79 cells.

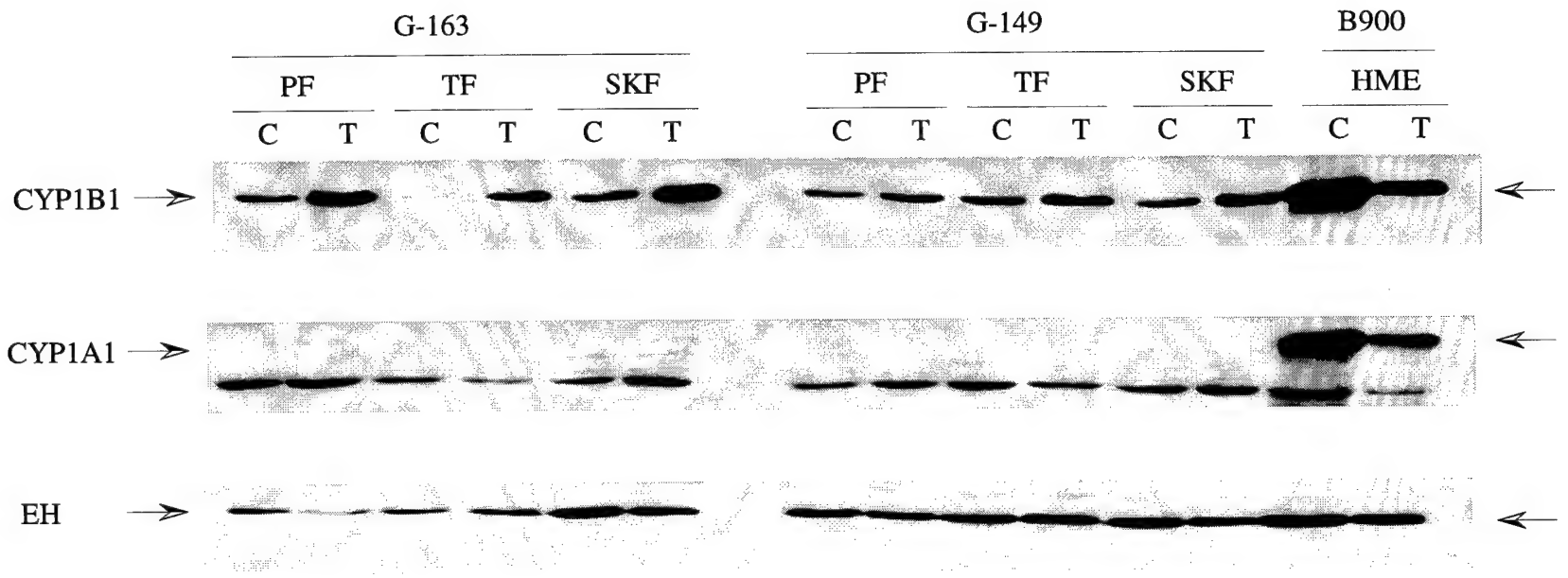


Figure 9. Expression of CYP1B1 and CYP1A1 in primary cultures of matched human mammary fibroblasts derived from normal peripheral (PF), tumor (TF) or skin (SKF) of two patients (G-163 & G-149). Microsomes were prepared from control (C) or 24 h TCDD-treated cells (T) at their 4th passage. Approximately 15 μg microsomal proteins were loaded on gels, and proteins on the gels were transfered to nitrocellulose membranes and probed with rabbit polyclonal antibodies to CYP1B1 and CYP1A1. Anti-rabbit IgG conjugated with horse raddish peroxidase was used as secondary antibody, and specific bands were visualized with enhance chemiluminescence (ECL) reagents kit. Microsomal extracts from human primary mammary epithelia (patient B900) is used as a positive control for both CYP1B1 and 1A1. Epoxide hydrolase (EH) which is a microsomal enzyme not affected by TCDD treatment was used as a loading control.

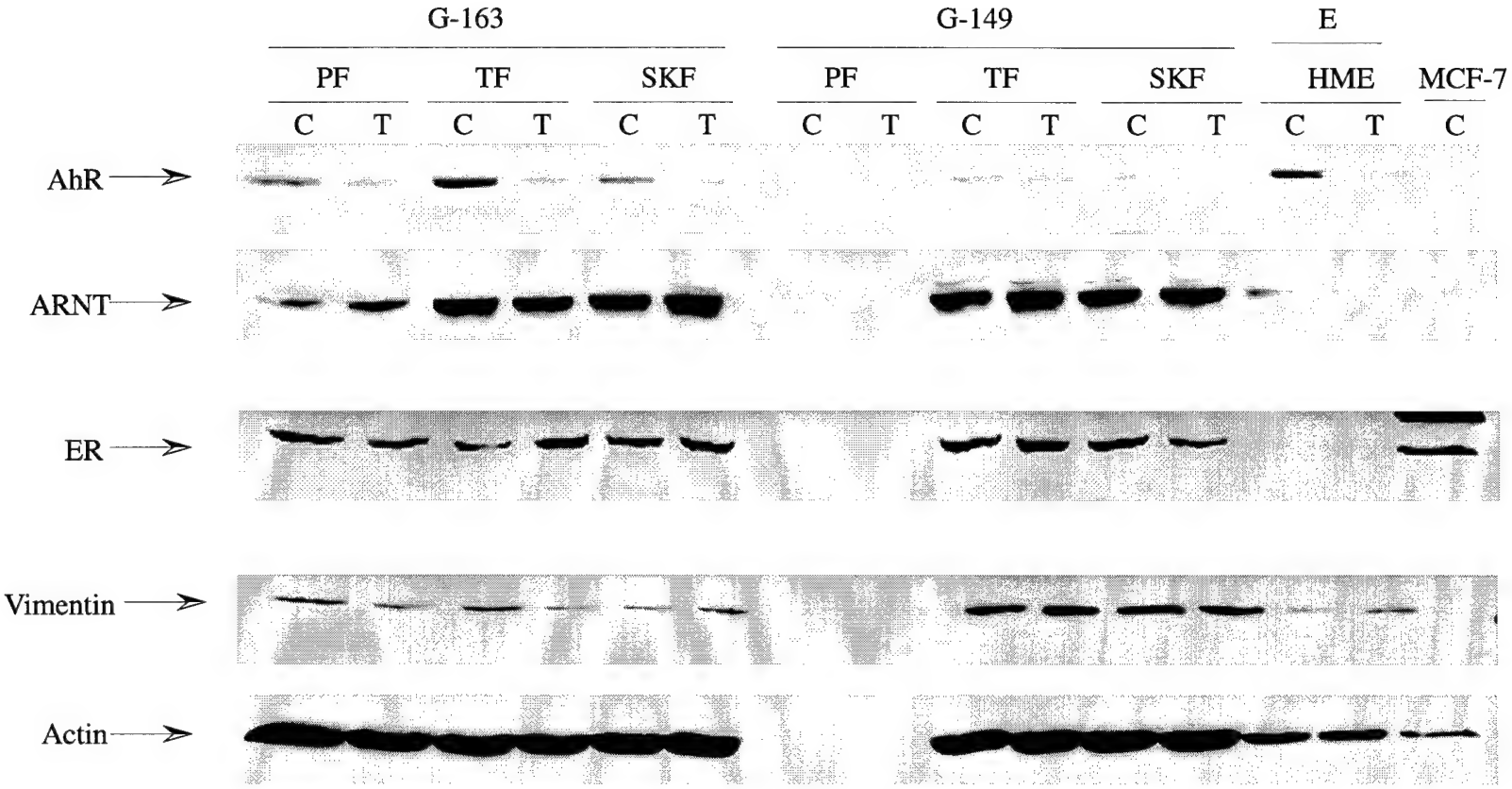
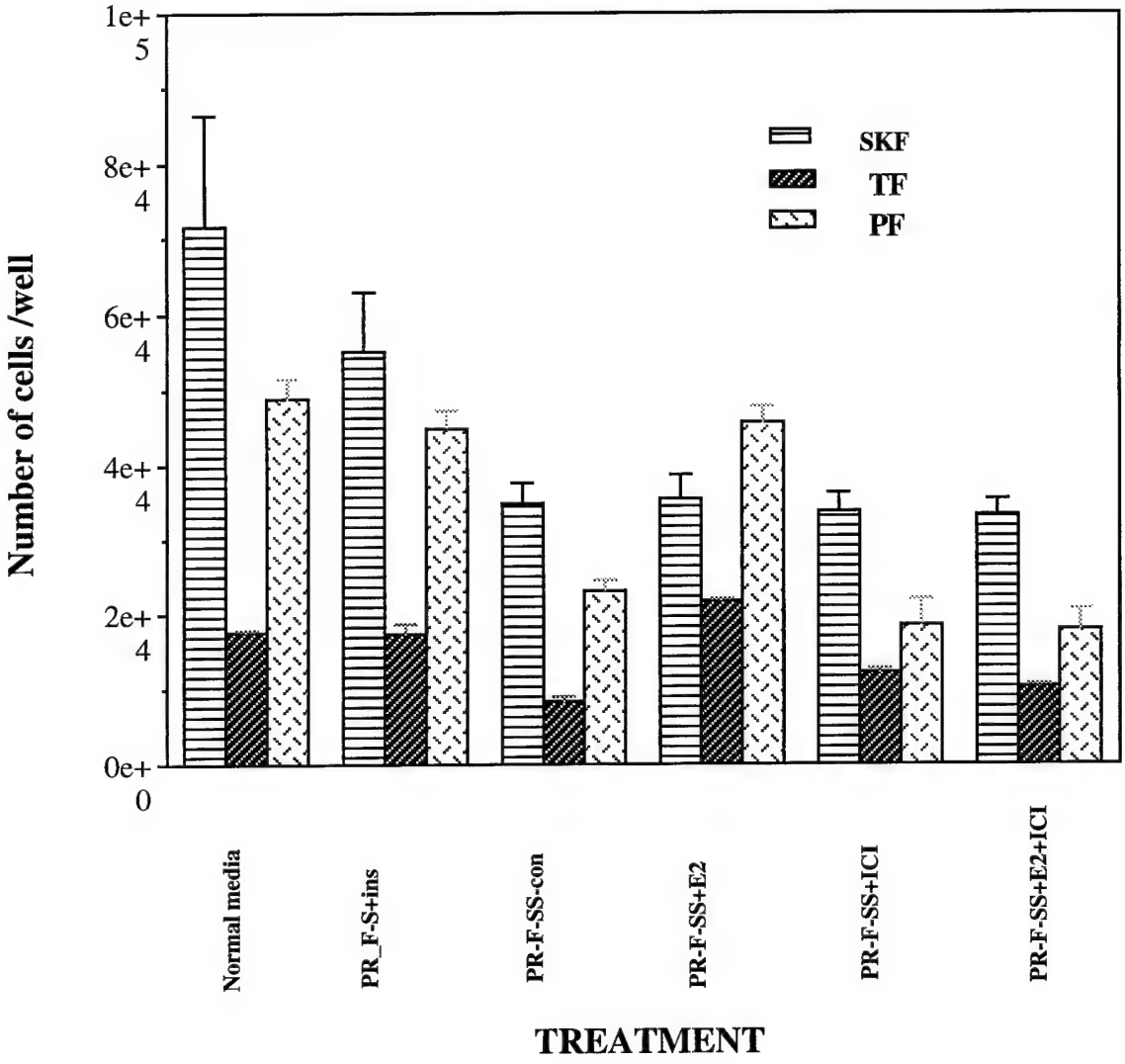


Figure 10. Expression of Ah receptor (AhR) and estrogen receptor (ER) in primary cultures of matched human mammary fibroblasts derived from normal peripheral (PF), tumor (TF) or skin (SKF) of two patients (G-163 and G-149). Total cellular proteins were isolated from Trizole® lysates of these cells after they were treated with 10 nM TCDD for 24 h. Approximately 25 μg microsomal proteins were loaded on gels, and proteins on the gels were transfered to nitrocellulase membranes and probed with rabbit polyclonal antibodies to AhR, ARNT, vimentin and ER, consecutively. Anti-rabbit or mouse IgG conjugated with horse raddish peroxidase were used as secondary antibodies, and specific bands were visualized with enhanced chemiluminescence (ECL) reagents kit. Extracts from normal primary human mammary epithelia (patient E) and MCF-7 human breast carcinoma cell line were used as controls. The blots membranes were probed for actin as loading control.

Figure 11. Cell proliferation of Human mammary Fibroblasts in response to estradiol treatment.

Fibroblasts were routinely maintaned in D-MEM/F12 media supplemented with 10% FBS and 10µg/ml insulin. Cells were pre-incubated in Phenol red-free media contaning charcoal-stripped-serum, without insulin before assigning the different treatment. Cells were kept in the different treatments for 48 h before collection and counting. Triplicate of each treatments and duplicate counting of each.



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THE ESTROGEN RECEPTOR DOES NOT DIRECTLY MODULATE INDUCTION OF CYP1A1 OR CYP1B1 EXPRESSION IN TWO HUMAN BREAST EPITHELIAL CELL LINES. W G R Angus, M Larsen, and C R Jefcoate. Department of Pharmacology and Environmental Toxicology Center, University of Wisconsin, Madison, WI.

The estrogen receptor (ER) has been implicated in the induction of CYP1A1 by TCDD. Using a quartet of complementary ER+/ER- cell lines representing earlier (T47D) and later (MDA-MB) stages of tumorigenesis and the "pure" antiestrogen ICI 182,780 (ICI), the role of the ER in TCDD induced expression of CYP1A1 and CYP1B1 message and protein was examined. T47D:A18 (ER+) represents a clonal expansion of T47D; T47D:C4:2W (ER-) was selected from A18 by growth in estrogen deficient media; MDA-MB-231 (ER-) is a classical model of ER- breast cancer; and MDA-MB-231/S30 (ER+) results from stable transfection of the wt hER into MDA-MB-231 cells. TCDD induced mRNA and protein expression of CYP1A1 and CYP1B1 to similar levels in both ER+ and ER- cells within a cell type, either in the presence or absence of ICI. The overall levels of expression were lower in MDA-MB-231 cells compared to T47D. Basal protein expression of CYP1B1 was greater in ER- cells than ER+ cells, while ICI treatment elevated the expression of basal CYP1B1 protein over that of vehicle alone in ER+ cells. ICI had no effect on ER- cells. These data indicate that ER does not directly influence TCDD induction of CYP1A1 or CYP1B1 at the mRNA or protein level. ER, however, plays an inhibitory role in the basal expression of CYP1B1 protein in these cells. Supported by grants DAMD17-94-J-4054 (CRJ) and NRSA 1-F32-ES05733-01 (WGRA).

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CYP1B1 REPRESENTS THE MAJOR PAH-RESPONSIVE P450 CYTOCHROME CONSTITUTIVELY EXPRESSED IN NORMAL PRIMARY HMEC. M Larsen, W G R Angus, P Brake, S Eltom, and C R Jefcoate. Department of Pharmacology and the Environmental Toxicology Center, University of Wisconsin, Madison, WI.

CYP1B1 and CYP1A1, the major PAH metabolizing P450 cytochromes, have been shown to metabolically activate 7,12-dimethylbenz(a)anthracene (DMBA) in a cell-type selective manner in rat mammary fibroblasts and rat mammary epithelial cells, respectively [Christou *et al.* (1995) *Molec. Cell. Endocrinol.* 115: 41-50.]. Conversely, constitutive CYP1B1 as well as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-inducible CYP1B1 and CYP1A1 expression has been identified in the transformed human mammary MCF-7 carcinoma cell line [Christou *et. al.* (1994) *Carcinogenesis* 15: 725-732.]. We have characterized CYP1B1 and CYP1A1 expression in early passage normal primary human mammary epithelial cells (HMEC) isolated from seven individuals. The primary cells demonstrated low constitutive levels of microsomal DMBA metabolism, which were highly inducible by TCDD. The regioselective distribution of DMBA metabolites generated by basal microsomes is consistent with human CYP1B1-mediated metabolism, while the profile of TCDD-induced metabolism suggests CYP1A1 activity. rtPCR and Northern analysis of RNA and immunoblot analysis of microsomal proteins each demonstrated low constitutive and increased TCDD-inducible expression of CYP1B1 in the normal primary HMEC. Constitutive CYP1A1 was only detectable in induced cell populations. CYP1B1 was expressed in two ER positive as well as two ER negative primary tumors by rtPCR, while CYP1A1 expression was essentially undetectable. AhR immunoblot analysis suggests that constitutive CYP1B1 expression parallels AhR expression in the HMEC. (Supported by NIEHS 144EN46)

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CYP1B1 REPRESENTS THE MAJOR PAH-RESPONSIVE P450 CYTOCHROME CONSTITUTIVELY EXPRESSED IN NORMAL PRIMARY HMEC and TUMOR-DERIVED TISSUES. M Larsen, W G R Angus, P Brake, S Eltom, and C R Jefcoate. Department of Pharmacology and the Environmental Toxicology Center, University of Wisconsin, Madison, WI.

CYP1B1 and CYP1A1, the major PAH metabolizing P450 cytochromes, have been shown to metabolically activate 7,12-dimethylbenz(a)anthracene (DMBA) in a cell-type selective manner in rat mammary fibroblasts and rat mammary epithelial cells, respectively [Christou *et al.* (1995) *Molec. Cell. Endocrinol.* 115: 41-50.]. Conversely, constitutive CYP1B1 as well as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-inducible CYP1B1 and CYP1A1 expression has been identified in the transformed human mammary MCF-7 carcinoma cell line [Christou *et. al.* (1994) *Carcinogenesis* 15: 725-732.]. We have characterized CYP1B1 and CYP1A1 expression in early passage normal primary human mammary epithelial cells (HMEC) isolated from seven individuals. The primary cells demonstrated low constitutive levels of microsomal DMBA metabolism, which were highly inducible by TCDD. The regioselective distribution of DMBA metabolites generated by basal microsomes is consistent with human CYP1B1-mediated metabolism, while the profile of TCDD-induced metabolism is representative of CYP1A1 activity. rtPCR and Northern analysis of RNA and immunoblot analysis of microsomal proteins each demonstrated low constitutive and increased TCDD-inducible expression of CYP1B1 in the normal primary HMEC which correlates with protein expression. Constitutive CYP1A1 was only detectable in induced cell populations. CYP1B1 was expressed in two ER positive as well as two ER negative primary tumors by rtPCR, while CYP1A1 expression was essentially undetectable. AhR immunoblot analysis suggests that constitutive CYP1B1 expression parallels AhR expression in the HMEC. (Supported by NIEHS 144EN46)

P450 and activities of microsomal ethoxyresorufin *O*-deethylase (EROD), methoxyresorufin *O*-demethylase (MROD), pentoxyresourfin *O*-dealkylase (PROD), a representative activity of P4501A1, P4501A2 and P4502B1/2, respectively, with dose-dependent manner. In contrast there was no effect on the P4502E1 catalyzed aniline hydroxylase. In the time-course experiment, methoxsalen exhibited a biphasic effect on EROD, MROD, and PROD activities, an initial inhibitory phase was followed by a phase of induction following a single treatment. Immunoblot analysis using anti-rat liver P4501A and P4502B revealed that increase in the apoprotein levels of P4501A1/2 and P4502B1/2 by methoxsalen was consistent with those in enzyme activity levels. Levels of mRNA of P4501A1/2 and P4502B1/2 were also increased by methoxsalen in Northern blot analysis. These results demonstrated that methoxsalen act as an inducer of the hepatic microsomal mixed function oxidase, selectively inducer of P4501A and P4502B families involved increases in mRNA levels. [Supported by KOSEF Grant 961-0505-117-2.]

677 MURINE *Cyp1a-1* INDUCTION IN MOUSE HEPATOMA HEPA-1C1C7 CELLS BY MYRISTICIN.

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Mouse hepatoma Hepa-1c1c7 (Hepa-1) cells were treated with myristicin to assess the role of myristicin in the process of the Cyp1a-1 induction. Treatment of Hepa-1 cells with myristicin increased the *Cyp1a-1* transcription in a dose-dependent manner as indicated by analysis of 7-ethoxyresorufin O-deethylase activity and Cyp1a-1 protein level and Cyp1a-1 mRNA. Myristicin, however, did not competitively displace [^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin from the Hepa-1 cytosolic aryl hydrocarbon (Ah) receptor in a competitive Ah receptor binding analysis using sucrose density gradient sedimentation and did not effect formation of DNA-protein complexes between the Ah receptor and its DRE target in a gel mobility shift assays using oligonucleotides corresponding to DRE 3 of the *Cyp1a-1*. These results suggest that the induction of the *Cyp1a-1* gene expression by myristicin in Hepa-1 cells might occur through an Ah receptor-independent pathway. [Supported by KOSEF Grant 961-0505-117-2.]

678 ROLE OF P450 4B1 IN 1,3-BUTADIENE (BD) AND BUTADIENE MONOXIDE (BMO) OXIDATIONS IN MOUSE TISSUES.

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Previously, we have shown that P450 2E1 is a major catalyst of BD oxidation to BMO in human liver microsomes, and that it is a major catalyst of BMO oxidation to diepoxybutane (DEB) in mouse liver microsomes. To further assess the role of 2E1 in BD and BMO oxidations in the mouse, we correlated BMO and DEB formations in male and female mouse liver, lung and kidney microsomes with the 2E1 marker activity, chlorzoxazone 6-hydroxylase. Because the results indicated no such correlation, the involvement of other P450s in BD and BMO oxidations in extrahepatic tissues was indicated. The BD oxidation pattern in male and female mouse kidney and lung microsomes was similar to 4B1 levels expressed in these tissues, as reported by Imaoka *et al.* To confirm the role of 4B1 in BD and BMO metabolism, inhibition experiments were conducted using antiserum to rabbit 4B1. When the antibody was used at a 2 mg IgG/mg microsomal protein in male mouse kidney and male and female lung, BMO formation was inhibited by 86%, 35%, and 40%, respectively. However, with male liver microsomes which have catalytic activity similar to those of male and female lung and male kidney, no inhibition was observed; the liver activity was only inhibited by 10% when the antibody concentration was doubled. Nearly complete inhibition of DEB formation from BMO was observed when the 4B1 antibody (2 mg IgG/mg protein) was included in kidney microsomal incubations. These results show that 4B1 is the major catalyst of BD and BMO oxidations in the male mouse kidney and that 4B1 also contributes to BD activation in male and female mouse lung. (Supported by NIH grant ES 06841).

679 SUPPRESSION OF CONSTITUTIVE AND INDUCIBLE CYTOCHROME P4501B1 BY GLUCOCORTICOIDS IN ISOLATED RAT MAMMARY CELLS.

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Cytochrome P4501B1 (CYP1B1) is expressed and regulated in a cell-specific manner by endogenous steroid and peptide hormones and ubiquitous environmental contaminants. We have demonstrated the presence of CYP1B1 in the rat and human mammary gland as the major constitutive polycyclic aromatic hydrocarbon (PAH)-metabolizing species. CYP1B1 and CYP1A1 exhibit cell-type specific expression in cultures of isolated rat mammary cells. A constitutively expressed CYP1B1 is stimulated by agonists of the Ah receptor in isolated rat mammary fibroblasts (RMF). In isolated rat mammary epithelial cells (RMEC), CYP1A1 is induced by TCDD; the presence of CYP1B1 in RMEC may be due to stromal contamination and is currently under investigation. Corticosterone and dexamethasone suppress both constitutive and TCDD-induced levels of CYP1B1 (50-60%) in both RMF and rat embryo fibroblasts (REF). This suppression is relieved by the glucocorticoid antagonist, RU486, suggesting a possible role for the glucocorticoid receptor. PAH induction of CYP1A1 is similarly suppressed in RMEC by a hormonal mixture containing progesterone and glucocorticoids. Glucocorticoid treatment does not affect translocation of the Ah receptor to the nucleus upon stimulation by TCDD. The activity of a luciferase construct containing the enhancer region of the mouse CYP1B1 promoter and transiently transfected into RMF and REF, was similarly suppressed (>50%) by glucocorticoids. Experiments are presented which document the steroid regulation of CYP1B1. Notably, we address whether steroid regulation is mediated through changes in Ah receptor expression and activity or through distinct *cis*-acting suppression elements in the CYP1B1 promoter. (PB Brake supported by NRSA Grant T32 ES07015 from the NIEHS. Supported by NIH Grant P30 CA14520 and DAMD Grant 17-94-J-4054).

680 ANALYSES OF THE 5'-FLANKING REGION OF THE MOUSE CYP1B1 GENE.

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Transcriptional activation of cytochrome CYP1B1 in rodents is stimulated by both polycyclic aromatic hydrocarbons and cAMP. It is expressed in steroidogenic tissues and embryonic cell lines with preferential expression in stromal fibroblasts relative to epithelial cells. *CYP1B1* is transcribed from a very compact gene that has two introns and three exons of which exon 2 is the start of the open reading frame. 1.75 kb upstream of the iniation codon ATG of a mouse genomic clone were subjected to DNA sequencing and deletion analyses linked to the reporter gene luciferase. DNA sequencing revealed 11 putative xenobiotic responsive elements (TnGCGTG) or (GCGTG), 4 GC-rich sequences that resemble DXE elements, in addition to 5 E-box elements and 3 steroidogenic factor-1 motifs. Primer extension identified a starting domain defining an exon 1 of 372 bp, substantially smaller than reported for human exon1 of CYP1B1. Segments containing exon 1 and 5'-flanking region were cloned upstream of the reporter gene luciferase. In transient transfection assays, TCDD induced the expression of luciferase 5-fold in the mouse C3H10T1/2 (express predominantly CYP1B1) when the CYP1B1 insert comprised exon 1 and 1 kb of 5'-flanking region. Similar induction was seen in Hepa1c1c7 cells (express predominantly CYP1A1) indicating the absence of the elements that confer the *in vivo* cell-specific expression. A 200 bp basal promoter region for mouse CYP1B1 has been identified that includes 2 putative Sp-1 sites, a TATA-box like site, and XRE sequences adjacent to the start site. TCDD induction is primarily dependent on positive regulatory elements present between -1193 and -1436 where three XREs are localized. An in vivo TCDD-induced hypersensitive site has been identified immediately on the 3'-side of this XRE cluster. A 30 bp oligomer containing one putative XRE (-1262 to -1231) was found to form complexes from nuclear extracts of C3H10T1/2 and Hepa1c1c7 cells. Negative regulatory elements have been identified in exon 1 (+1 to -300) and between the promoter and TCDD enhancer regions. Supported by NIH grant P30 CA 14520.

681 REGULATION OF CYP2B EXPRESSION BY SQUALESTATIN 1, AN INHIBITOR OF SQUALENE SYNTHASE, IN PRIMARY CULTURED RAT HEPATOCYTES.

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We recently reported that several inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting step in the biosynthesis of sterols and other isoprenoids, induced CYP2B, CYP3A and CYP4A mRNA and immunoreactive protein when incubated with primary cultures of rat hepatocytes. To examine the effects of inhibition of only sterol biosynthesis on P450 expression, we have utilized the drug squalestatin 1 (SQ1), a potent inhibitor of squalene synthase, the first committed step in sterol biosynthesis.

The Estrogen Receptor Influences Expression of CYP1A1 and CYP1B1 In Opposing Directions in Human Breast Cancer Cell Lines By Indirect Pathways

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[IV]Abbreviations: CYP, cytochrome P450; PAH, polycyclic aromatic hydrocarbon; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; ER, estrogen receptor; DMBA, 7,12-dimenthylbenz(a)anthracene; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator protein; ICI, ICI 182,780; ECL, enhanced chemoluminescence; $T47D^+$, T47D:A18 cells; $T47D^-$ T47D:C4:2W cells; MDA^+, S30 cells; MDA^-, MDA-MB-231 cells.

Abstract

The estrogen receptor (ER) has been implicated in the induction of CYP1A1 by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in human mammary cells. The impact of a stable ER deficient phenotype on TCDD-induced expression and activity of CYP1B1, in comparison to CYP1A1, has been examined by utilizing a quartet of complimentary ER^+/ER^- human breast epithelial cell lines representing earlier (T47D) or later (MDA-MB-231) stages of tumorigenesis. The effects of an acute loss of ER were evaluated using the antiestrogen ICI 182,780 (ICI). CYP1B1, but not CYP1A1, was observed under basal conditions in all cell lines, and TCDD induced both CYP1A1 and CYP1B1 mRNA and protein in all ER^+ and ER^- clones. Basal and TCDD-induced CYP1B1 expression as well as TCDD-induced CYP1A1 expression (mRNA and protein) were all substantially greater in T47D cells than MDA cells, irrespective of ER status. Further, for each cell type, the ER^- phenotype was associated with a lesser TCDD-induced CYP1A1 expression (protein > mRNA) but a greater basal and induced CYP1B1 expression (mRNA > protein). ICI-mediated 48 hour loss of ER activity in ER^+ cells did not reproduce the changes in CYP1A1 and CYP1B1 expression observed with stable loss of ER. Changes in the extent of DMBA metabolism between the cell lines was consistent with differences in CYP1A1 and CYP1B1 expression. In the T47D lines, the majority of basal microsomal metabolism of DMBA was due to CYP1B1, based on regioselective metabolite distribution and inhibition by anti-CYP1B1 antibodies (>70%). Metabolism in TCDD-induced microsomes was mostly due to CYP1A1, and was inhibited by anti-CYP1A1 antibody (>50%). Although MDA lines demonstrated negligible basal microsomal DMBA metabolism, TCDD-induced MDA^+ cells demonstrated CYP1A1 activity, and TCDD-induced MDA^- cells displayed CYP1B1 activity. Ah receptor levels were approximately 10-fold greater in MDA cells than T47D, irrespective of ER status. However, ARNT levels were unaffected by cell type or ER status. The expression of CYP1A1 and CYP1B1 may depend on the extent

of AhR expression, but is more highly dependent on undefined regulatory factors that distinguish T47D cells from MDA cells. We conclude that the expression of CYP1A1 and CYP1B1 is indirectly modulated in opposite directions by the presence/absence of the ER through a gradual reorientation of cell signalling.

Introduction

Cytochrome P4501B1 (CYP1B1)[IV] is found in tissues strongly influenced by steroids, such as the breast, uterus and prostate (1,2). Like its familial relative, cytochrome P4501A1 (CYP1A1), CYP1B1 can be induced by polycyclic aromatic hydrocarbons (PAHs) through a mechanism involving the aryl hydrocarbon receptor (AhR) (3,4). Tetrachlorodibenzo-p-dioxin (TCDD), the archetypal agonist for AhR-mediated gene induction, induces the expression of CYP1B1 and CYP1A1 in human breast epithelial MCF7 cells, although CYP1B1 alone is expressed constitutively (3). CYP1B1 is also present basally in normal human breast epithelial cells, while CYP1A1 is below the lower limit of detectability or present at very low levels (<0.03 to 0.16 pmol/mg microsomal protein) (5). Functional involvement of CYP1B1 in PAH metabolism has been demonstrated in MCF7 cells (3). Metabolism of the potent rodent mammary carcinogen 7,12-dimethylbenz (a) anthracene (DMBA)(6-9) by microsomes from uninduced MCF7 cells resulted in a different metabolite distribution from that observed for CYP1A1. Anti-CYP1B1 antibodies, but not anti-CYP1A1 antibodies were inhibitory (3). By contrast, microsomes from TCDD-induced MCF7 cells displayed a CYP1A1 pattern of DMBA metabolites, which was completely inhibited by anti-CYP1A1 antibodies. DMBA, like other PAHs, requires metabolic activation through P450 cytochromes to a dihydrodiol epoxide to initiate carcinogenesis (10-12).

Human CYP1B1 has been linked to the metabolism of 17ß-estradiol into 4-hydroxyestradiol (13,14) and the bioactivation of many PAHs. A natural substrate for CYP1B1 has important physiological functions, since the expression of CYP1B1 is under hormonal control in many steroidogenic tissues, CYP1B1 displays selective expression in the mouse embryo [D.L. Alexander, personal communication], and there is a reported linkage of mutations to a developmental disease (congenital glaucoma) (15). Thus, elevations of estradiol 4-hydroxylation in breast and uterine tumors relative

to surrounding tissues indicates that there may be an elevation of functional CYP1B1 in these tumors. This suggests a possible role for CYP1B1 in tumorigenesis in addition to mutagen activation (16).

Some commonly used human mammary cell lines, including MCF7 and T47D cells express the estrogen receptor (ER^+) (17,18), while others, such as MDA-MB-231, lack the receptor completely (ER^-) (19). The ER has been implicated in the induced expression and activity of CYP1A1 through a deficiency in AHH activity (a measure of CYP1A1 activity) in ER-negative MDA-MB-231 cells, which was restored by transient transfection of ER into these cells (20). Another report has demonstrated a lack of TCDD-inducible AHH (CYP1A1) activity in several additional ER^- breast tumor cell lines, regardless of their capacity to bind TCDD (21). It was concluded that ER status plays an important role in the TCDD-induction of CYP1A1 in human breast epithelial cells. However, we have recently observed TCDD-induction of CYP1A1 and CYP1B1 mRNA in primary human breast epithelial cells which are ER^- (5), suggesting that the ER is not directly connected to TCDD-induction in human breast cells. Recent studies further suggest that unusually low levels of Ah receptor nuclear translocator protein (ARNT) in some cell lines may also contribute to low CYP1A1 induction (22).

We have tested the role of the ER in CYP1A1 and CYP1B1 expression by utilizing pairs of ER^+/ER^- cells. The T47D:A18 ($T47D^+$), which contains the ER, was paired with an ER^- clone (T47D:C4:2W, $T47D^-$), derived from $T47D^+$ by long-term culture in estrogen-free medium (23). MDA-MB-231 (MDA^-) cells, which lack ER, were paired with a clone resulting from stable transfection of the ER into MDA^- cells (S30, MDA^+) (24). Both of the T47D cell lines depict a classic epithelial cell morphology, while the MDA cells are morphologically fibroblast-like, representative of a more advanced malignant and metastatic nature. $T47D^+$ cells are growth-stimulated by estrogen, but the MDA^+ cells are growth inhibited, indicating that the transfected ER functions differently from the native ER in non-transfected cells (24). The selection of

ER^- cells which are insensitive to estrogens when the ER is reintroduced therefore likely involves slow compensatory and adaptive changes in growth factor signalling pathways, other than those controlled by ER. The effect of a rapid loss of ER activity has been examined through treatment of the ER^+ lines with the "pure" antiestrogen ICI 182,780. These combinations of cells and treatments have been used to evaluate whether ER status or other aspects of the cell phenotype are important in determining CYP expression. Since changes in CYP expression may be mediated by differences in levels of AhR and ARNT proteins, we have also examined the expression of these proteins under the same conditions. In these studies, we demonstrate that CYP1B1 and CYP1A1 exhibit opposing sensitivities to the ER status of the cell.

Materials and Methods

MDA-MD-231 cells (19) were purchased from ATCC. The cell lines T47D:A18, T47D:C4:2W, and S30 were generous gifts from Drs. Craig Jordan and John Pink (Northwestern University, University of Wisconsin Comprehensive Cancer Center)(23,24). ICI 182,780 was obtained from Dr. Alan Wakling (Zenica Pharmaceuticals, Manchester, England). The human CYP1B1 antibody used in these studies was a generous gift from Dr. Craig Marcus (University of New Mexico) and Dr. William Greenlee (University of Massachuttes); the Ah receptor monoclonal antibody and ARNT polyclonal antibody were obtained from Dr. Gary Perdew (Penn State University). Antibody to estrogen receptor was purchased from Santa Cruz Biologicals (HC-20; Santa Cruz, CA). The antibodies for CYP1A1, and inhibitory antibodies for CYP1A1 and CYP1B1 were generated in this laboratory. TCDD was purchased from Accustandard, Inc. (New Haven, CT). a-^{32}PdCTP was purchased from NEN Dupont (Boston, MA). Reagents purchased from GIBCO (Grand Island, NY) included Dulbecco's Modified Eagle Medium/Ham's F12 (DMEM/F12), RPMI 1640, and agarose. Cell culture was carried out using Falcon flasks and Corning plates. Fetal bovine serum (FBS) was obtained from Gemini (Calabasas, CA) and dextran charcoal

stripped FBS was purchased from HyClone (Logan, UT). Reagents purchased from the Sigma Chemical Co. (St. Louis, MO) included glucose-6-phosphate, 7,12-dimethylbenz (a)anthracene, nicotinamide dinucleotide phosphate (NADP), HEPES, insulin, ammonium persulfate, TEMED, dimethylsulfoxide (DMSO), phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, soybean trypsin inhibitor, aurin tricarboxylic acid (ATA), salmon sperm DNA, diethyl polycarbonate (DEPC), penicillin/streptomycin solution and phenol red-free DMEM/F12. Sodium dodecyl sulfate (SDS) was purchased from BioRad, Inc. (Hercules, CA). Proteinase K was obtained from Fisher Chemical (LaJolla, CA). Formamide was purchased from Ambion (Austin, TX). Oligo dT was obtained from Calbiochem (LaJolla, CA). The PRIME IT II kit was purchased from Stratagene (LaJolla, CA). Nylon (Hybond N^+) and nitrocellulose membranes (Hybond ECL), Hyperfilm ECL film, and ECL reagents were obtained from Amersham (Arlington Heights, IL). BCA protein determination kit was purchased from Pierce (Rockford, IL). Other reagents used for these studies were of the highest grade possible.

Cell Culture

All cells were maintained in monolayer culture in 75 cm^2 or 175 cm^2 flasks in a humidified 5% CO_2 atmosphere. MDA^- cells were grown in DMEM/F12 with 10 mm HEPES, 5% FBS, and penicillin/streptomycin. The MDA^+ cells, which are growth inhibited by estrogens (24), were grown in phenol red-free DMEM/F12 containing 15 mm HEPES and 5% dextran charcoal stripped FBS. T47D+ and T47D- cells were cultured in phenol red-free RPMI 1640 with 10% FBS and penicillin /streptomycin. All cells were passaged using 0.5% trypsin at 80-90% confluence.

Hybridization analysis of mRNA

Cells cultured in 150 mm plates were treated or not for 24 hours with 10^{-7} M ICI 182,780, then for an additional 24 hours with or without 10^{-8} M TCDD in the continued presence or absence of ICI. Controls were treated for 24 hours with dimethylsulfoxide

(DMSO, 0.1%). Cells were harvested and poly A^+ RNA isolated according to the technique of Badley *et al.*, 1988 (25). Briefly, cells were washed with PBS (0.01 M phosphate buffer in 2.7 mM KCl and 137 mM NaCl, pH 7.4) containing 20 μM ATA and lysed in a buffer consisting of 0.2 M NaCl, 0.2 M Tris-HCl, pH 7.5, 0.15 mM $MgCl_2$ 2% SDS, 200 μg/ml Proteinase K, and 20 μM ATA. Lysates were sheared with a 23 gauge needle and incubated for 2 hours at 45 C°, then agitated with oligo dT. Poly A^+ RNA was eluted with five 300 μl aliquots of DEPC-treated water. Eluted RNA was precipitated by addition of 2 volumes of 100% ethanol, and chilling for 2 hours at -80 C°. Precipitated RNA was washed with 70% ethanol and air dried. The RNA was resuspended in sterile DEPC-treated H_2O, the 5 fractions pooled, and quantitated by reading absorbance at 260 nm.

Poly A^+ RNA was electrophoresed through a 1% formaldehyde-containing agarose gel. Capillary action was used to transfer the RNA to nylon membrane, and was crosslinked to the membrane by UV. Membranes were prehybridized in a buffer of 6X SSC, 5X Denhardt's reagent, 0.1% SDS, 10 μg/ml salmon sperm DNA, and 50% formamide at 42 C° for at least 2 hours. Membranes were then incubated with probes for ß-actin, human CYP1A1, and human CYP1B1 as described below. Probes were randomly labeled using a-^{32}PdCTP (50 μCi) following the manufacturer's instructions for the Prime-It II kit. Specific activity was 10^6 cpm or greater per ml of probe. Nonspecific hybridization was removed by sequential washing with 2X SSPE + 0.5% SDS, 1X SSPE + 0.5% SDS, 0.5X SSPE + 0.5% SDS, and 0.25X SSPE + 0.25% SDS at hybridization temperature for 15 min. Signals were visualized by autoradiography and quantitated by densitometry.

Microsomal metabolism of DMBA

Microsomes were generated by differential centrifugation as previously described (26) from $T47D^+$, $T47D^-$, MDA^-, and MDA^+ cells treated with or without ICI 182,780 for 24 hours, then with or without TCDD for an additional 24 hours in the

presence/absence of ICI. Microsomal metabolism was performed using 0.2 to 1 mg of TCDD-induced and basal microsomal protein, respectively, as described by Christou, *et al.*, 1991 (3). Incubations were 15 min at 37 C°, and 1.5 μM DMBA was used in a 1 ml volume reaction. Inhibition studies were carried out using chicken anti-CYP1B1 (IgY) at 5 mg/mg microsomal protein or rabbit anti-CYP1A1 (IgG) antibodies (3) at 10 mg/mg microsomal protein. The reaction mixtures containing the antibodies and preimmune serum were incubated at room temperature for 40 min prior to the 15 min incubations with DMBA at 37 C°.

Cell lysates and Western Immunoblots

Confluent cells in 60 mm plates, treated as previously described for ICI and TCDD, were harvested by scraping in PBS and pelleted. A lysis buffer consisting of 20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM sodium orthovanadate, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml soy bean trypsin inhibitor, and 1 mM PMSF was added to each pellet. Following 10 min on ice, samples were triturated using a pipette tip and centrifuged at 10,000 g for 30 min at 4 C°. Supernatants were transferred to new tubes, an aliquot removed for protein determination, and frozen at -80 C°.

Cellular proteins were separated on SDS-PAGE containing 7.5% acrylamide as per Laemmli (1970) (27). Proteins were transferred to nitrocellulose membrane for 2 hours at 0.4 A. Transfer was confirmed by staining with Ponceau's S stain. Primary antibodies were incubated for 2 hours, and secondary antibodies (1:10,000 dilutions) were incubated for 30 min. Reactive proteins were visualized using the ECL procedure according to manufacturer's instructions.

Protein levels in cellular extracts and microsomal preparations were determined using the BCA method as per manufacturer's instructions using bovine serum albumin as a standard.

Results

The expression of CYP1A1 and CYP1B1 mRNA, protein and DMBA metabolism has been examined in 2 pairs of human mammary cell lines, each pair providing a comparison between ER^+ and ER^- status ($T47D^+$, $T47D^-$, MDA^+, MDA^-). Cells have been examined under basal conditions, and following a 24 hour treatment with TCDD.

mRNA Expression

Northern hybridization of mRNA showed no basal expression of CYP1A1 message, however, treatment with TCDD for 24 hours induced CYP1A1 gene expression in all 4 lines (Figure 1A). Overall, the amount of CYP1A1 mRNA in TCDD-induced T47D cells was greater than in corresponding TCDD-induced MDA cells ($T47D^+/MDA^+ = 2$; $T47D^-/MDA^- = 4$).

The expression of CYP1B1 mRNA was observed under basal conditions in each of the 4 lines and was induced by TCDD (Figure 1B). This basal CYP1B1 mRNA expression was much less in ER^+ lines than in ER^- lines ($T47D^+/T47D^- = 0.1$; $MDA^+/MDA^- = 0.4$). TCDD induced CYP1B1 mRNA in T47D cell lines by an average of 7-fold, and in MDA cells by about 4-fold. Thus, TCDD-induced CYP1B1 mRNA is greater in ER^- cells from both cell lineages.

Protein Expression

Immunodetectable CYP1A1 protein was also only observed in TCDD-treated samples (Figure 2A). Consistent with mRNA measurements, T47D cells contained substantially more CYP1A1 protein than MDA cells ($T47D^+/MDA^+ = 10$, $T47D^-/MDA^- = 100$) (MDA^- cells express barely detectable levels of CYP1A1, not observable on the graph; <1 relative unit). It can be seen that differences in protein expression are much greater than differences observed at the level of mRNA. For both T47D and MDA cell types, ER^+ lines contained greater amounts of TCDD-induced CYP1A1 protein than their ER^- partners ($T47D^+/T47D^- = 6$, $MDA^+/MDA^- = 7$). Again for the MDA lines this ratio (MDA^+/MDA^-)is much greater for protein than for mRNA.

Expression of CYP1B1 protein in the 4 lines largely paralleled the relative mRNA levels (Figure 2B). Basal expression of CYP1B1 protein was generally greater in T47D lines compared to the equivalent MDA line ($T47D^+/MDA^+ = 3$; $T47D^-/MDA^- = 7$), and for both cell lineages, the ER^+ clone expressed lower levels of protein than the ER^- clone ($T47D^+/T47D^- = 0.2$; $MDA^+/MDA^- = 0.6$). MDA^- cells contained low but clearly discernable basal CYP1B1, while in MDA^+ cells expression was scarcely detectable. TCDD- induction of CYP1B1 protein closely paralleled induction of mRNA expression, with a 5-10-fold induction in each cell type. Following TCDD induction, $T47D^-$ and MDA^- expressed similar amounts of CYP1B1 protein, with MDA^+ expressing about 5 times less, and $T47D^+$ expressing about 2 times more.

Functional Activity

The functional activity of CYP1A1 and CYP1B1 from cellular microsomal protein has been calculated relative to recombinant CYP1A1 and CYP1B1 protein standards as shown in Table 1. A ratio of 5,6-dihydrodiol to 8,9-dihydrodiol was used as a reference parameter to determine if the metabolism pattern was CYP1A1-like or CYP1B1-like. Based on metabolism by the recombinant human cytochrome, a ratio of 1.0-1.5 indicated a pure CYP1B1 activity, while a ratio of 0.2-0.3 was typical for CYP1A1. A higher ratio of 10,11/8,9 dihydrodiols also typified CYP1B1 rather than CYP1A1 activity (1.0 versus 0.1).

Consistent with the measurement of cytochrome P450 protein expression, the T47D lines had a much greater DMBA metabolizing activity than the equivalent MDA cells (basal: $T47D^+/MDA^+ = 8.5$, $T47D^-/MDA^- = 6$; TCDD-induced: $T47D^+/MDA^+ = 13$, $T47D^-/MDA^- = 10$; Table 1). Under basal conditions, T47D cells displayed a DMBA metabolite pattern that was similar to, but not identical to that seen for recombinant CYP1B1 ($T47D^+$ 5,6-/8,9- = 0.6, $T47D^-$ 5,6-/8,9- = 0.5). Induction by a 24 hour TCDD treatment shifted the metabolite pattern to a pure CYP1A1 distribution ($T47D^+$ 5,6-/8,9- = 0.25, $T47D^-$ 5,6-/8,9- = 0.27). Under basal conditions the $T47D^-$ cells had a 50%

greater activity than the T47D$^+$ cells, again consistent with the greater expression of CYP1B1 protein and mRNA in the ER$^-$ cells. However, the TCDD-induced metabolic activity of the T47D$^+$ cells was 3 times greater than that of T47D$^-$ cells, which parallels the relative expression of the much more active CYP1A1 protein (5). The MDA lines exhibited much lower DMBA-metabolizing activity than the T47D lines. Neither MDA$^+$ or MDA$^-$ cells showed significant basal activity, but a CYP1B1-like metabolic pattern was observed in the TCDD-induced MDA$^-$ cells (5,6-/8,9- = 0.5), while TCDD-treated MDA$^+$ cells demonstrated a CYP1A1-like pattern of metabolism (5,6-/8,9- = 0.23). Again, these patterns were consistent with the selectivity of CYP protein expression.

Inhibitory antibodies to the P450s were used to determine the contribution of CYP1A1 and CYP1B1 to the DMBA metabolism profile in T47D$^+$ cells (Table 2). Under basal conditions, the anti-CYP1B1 antibody inhibited overall metabolism by 65%, while the anti-CYP1A1 antibody had no effect. In contrast, the anti-CYP1A1 antibody inhibited greater than 50% of the TCDD-induced DMBA metabolism (Table 2). Examination of the distribution of metabolites removed by anti-CYP1B1 was evaluated by subtraction of anti-CYP1B1 metabolite values from preimmune metabolite values. This indicated that ratios of dihydrodiols were very similar to those found for recombinant hCYP1B1 (1.0 for 5,6-/8,9-dihydrodiols; 0.4 for 10,11/8,9 dihydrodiols). The remaining metabolites that are unaffected by either antibody indicate a residual basal metabolic activity that may be due to other P450s present in breast epithelial cells (28) or to a peroxidative mechanism.

Specific Enzyme Content and Specific Activity

Specific P450 content per mg cellular protein for each enzyme was determined by regression analysis of relative intensity values (densitometry readings) against a standard curve generated utilizing recombinant proteins (Table 3). T47D cells contained greater specific CYP1A1 than MDA cells (T47D$^+$/MDA$^+$ = 4; T47D$^-$/MDA$^-$ = 38), while the ER$^+$ clones contained a greater specific CYP1A1 content tan their ER$^-$

counterparts ($T47D^+/T47D^- = 7$; $MDA^+/MDA^- = 60$). The specific content of CYP1B1 was also greater in T47D cells than MDA cells (basal: $T47D^+/MDA^+ = 3$, $T47D^-/MDA^- = 7$; induced: $T47D^+/MDA^+ = 7$, $T47D^-/MDA^-$ _ 1), but generally, greater in the ER^- clones than in the ER^+ clones (basal: $T47D^+/T47D^- = 0.2$, $MDA^+/MDA^- = 0.6$; induced: $T47D^+/T47D^- = 2$, $MDA^+/MDA^- = 0.4$). These specific content values agree with functional activity levels for each cell type.

A turnover value (P450/hr) was calculated for CYP1A1 and CYP1B1 in each line based on the specific enzyme content, the total DMBA metabolizing activity, and the fractions of total activity inhibited by specific antibodies under both basal and induced conditions (Table 3). The MDA lines had substantially lower CYP turnover than the T47D lines. MDA^- cells contained vanishingly low levels of CYP1A1, hence a turnover value was unquantifiable. In the T47D lines, the turnover values for each enzyme support the hypothesis that basal metabolism is mediated by CYP1B1, which switches to CYP1A1 with TCDD induction. Further, in the MDA lines, the turnover values confirm the limited CYP1A1 expression in these cells, and that CYP1B1 is the predominant metabolic enzyme for PAHs in these cells.

Effects of Antiestrogen and Expression of Estrogen Receptor

Levels of ER protein in the 4 lines were verified by immunoblotting (Figure 3). As expected, MDA^- and $T47D^-$ cells expressed no ER protein, while the MDA^+ expressed 2.5-fold more ER than the $T47D^+$. Treatment with ICI 182,780 for 48 hours decreased the amount of ER in both the $T47D^+$ and MDA^+ cells by 70%.

Treatment of the ER^+ cell lines with ICI had little effect on CYP1A1 message or protein (Figure 4A; Table 4). Treatment with ICI also had minimal effects on CYP1B1 mRNA and protein (Figure 4B; Table 4). Thus, this 48 hour treatment with ICI failed to reproduce the differences in CYP expression observed between ER^+ and ER^- lines.

Ah Receptor and ARNT Expression

Ah receptor, which mediates the induction of CYP1A1 and CYP1B1 by TCDD,

was present in all 4 lines (Figure 5). The AhR levels in the MDA lines was about 10-fold greater than in the T47D lines. The presence or absence of the ER did not affect the level of AhR. The apparently lower levels of AhR in the $T47D^-$ cells compared to $T47D^+$ cells, which could be suggestive of an ER effect, was not supported by comparison of MDA^- cells to MDA^+ cells, hence the lowered AhR levels in the $T47D^-$ is likely an inherent property of the cells. (Using the TRIzol (GIBCO) method for obtaining proteins, $T47D^-$ cells again demonstrate lowered AhR levels. WGRA, unpublished observations) The 24 hour TCDD treatment decreased the amount of AhR in each cell line, consistent with previous observations on mouse hepatoma cell lines that TCDD induces a translocation of the receptor to the nucleus followed by downregulation (29).

Levels of ARNT protein, which plays a role in the translocation of AhR across the nuclear membrane and forms a heterodimer with AhR necessary for binding to xenobiotic response elements, were similar in both T47D and MDA cell lines. No apparent effect of the ER on ARNT protein expression was observed. Further, TCDD treatment had no effect on levels of ARNT protein in any of these cell lines (Figure 5).

Discussion

These studies have characterized the expression of CYP1A1 and CYP1B1 in two pairs of ER^+ and ER^- cell lines which were derived from T47D and MDA-MB-231, and which represent earlier and later stage tumor cells, respectively. We show that CYP1B1 is expressed in the absence of an external stimulus, whereas CYP1A1 is only expressed after treatment with TCDD, the most potent stimulator of the Ah receptor. CYP1B1 is also stimulated by TCDD, but by a smaller factor than CYP1A1. This pattern of expression has also been observed in other cell lines which express CYP1A1 and CYP1B1 (3).

The current studies indicate that stable loss of the ER is associated with opposing expression and activity of CYP1A1 and CYP1B1. CYP1A1 expression was

greater, and CYP1B1 expression was lower, in the ER^+ clones of both cell types. This pattern was observed both at the mRNA and protein levels, and was reflected in the DMBA metabolic activity profiles. These ER-associated effects on the expression and activity were superimposed on the generally lower expression of CYP1A1 and CYP1B1 in the MDA lines as compared to the T47D lines. This difference was observed with both the basal and TCDD-induced expression of these genes, and indicated a strong effect of an ER-independent factor which appears to emerge as cells loose the ER and/or progress to later stage tumors.

The contributions of CYP1A1 and CYP1B1 to the metabolism of the prototypical PAH, DMBA, was resolved by determining differences in regioselectivity of DMBA metabolites and selective antibody inhibition. Consistent with the expression profiles, CYP1B1-like metabolic activity predominated under basal conditions, as previously reported for MCF7 cells (3) and as reported elsewhere for normal human mammary epithelial cells (5). The strong suppression of CYP1A1 expression in MDA^- cells resulted in TCDD-induced activity which was almost entirely attributable to CYP1B1. In the other cell lines most of the TCDD-induced activity was due to CYP1A1, even though CYP1B1 protein levels were comparable to those of CYP1A1. This incongruity can be rationalized based on the 6-fold greater activity of CYP1A1 (5).

An acute loss of the ER following 48 hours of ICI treatment (30,31) of ER^+ clones did not convert the expression patterns of CYP1A1 and CYP1B1 over to the different expression patterns observed in ER^- clones. This observation indicates that while the presence of absence of the ER influences expression of CYP1A1 and CYP1B1, the regulatory pattern is not a direct result of transcriptional regulation of these genes by ER.

Levels of the Ah receptor, which mediates PAH induction of CYP1A1 (32) and CYP1B1 (3,4) may also influence CYP1A1 and CYP1B1 expression. The approximately 10-fold differences in AhR protein expression between T47D- and

MDA-derived lines were inversely related to mRNA and protein expression of CYP1A1 or CYP1B1. Further, there was no effect of ER status on AhR expression in either cell type. Additionally, amounts of ARNT protein were not different between T47D and MDA cells, regardless of ER status. The lower AhR content in $T47D^-$ cells compared to $T47D^+$ appears to be an inherent property of the cells and not related to ER status, since in the MDA lines, there is no significant difference in AhR expression between the ER^+ and ER^- clones. [An alternate method for obtaining cellular proteins (TRIzol, GIBCO, as per manufacturer's instructions) provided data identical to those presented here.]

With respect to the inverse relationship between AhR content and CYP expression, the levels of ARNT protein do not appear to be rate limiting in the MDA lines. Further, the lower levels of AhR in the T47D cells appears to be quite sufficient to elicit a maximal induction response to TCDD in these cells. The excess AhR expression in the MDA lines, therefore, may be tying up or interfering with (an)other protein(s) which do participate in CYP expression.

Aside from ER status and AhR/ARNT expression, the backgrounds of T47D and MDA cells differ significantly (18,19), with respect to factors relating to tumor progression. These additional factors may be important for expression of CYP1A1 and CYP1B1. The ER^+, hormone dependent, morphologically epithelial cell shaped T47D line was used as a model of an early stage tumor. These cells express keratins and casein proteins (18,33,34) similarly to normal mammary luminal epithelial cells. We used the ER^-, hormone independent, morphologically fibroblast shaped MDA^- cells as a model of an aggressive, later stage tumor. These cells express vimentin and CD44 (34) -- markers for fibroblasts, and suggestive of dedifferentiation. The estrogen-independent growth of MDA^- cells suggests that another mitogenic ligand/receptor pathway has taken over the mitogenic functions previously filled by estrogen and the ER. Possibly the heregulin/ErbB receptor pathway could fulfill this role, since MDA^-

cells produce heregulin and contain ErbB receptors (35). Such mitogenic reprogramming may be just one of many systematic alterations occurring in cells stably lacking ER. For instance, T47D$^-$ cells, which were derived from T47D by long term growth in estrogen-free medium over a period of greater than 8 months, have had the time to reprogram their mitogenic pathways to respond to ER-independent growth stimuli in a manner similar to the MDA$^-$ cells. MDA$^+$ cells, which are stably ER transfected, are growth inhibited by estrogens. This phenomenon has also been observed in other ER$^-$ lines stably transfected with ER (36,37). Thus it appears that following the reintroduction of the ER, the cell's mitogenic machinery remains under the influence of the reprogrammed, compensatory mitogenic pathway(s). The reintroduced ER is not only mitogenically ineffective, but may even inhibit cell growth.

From the current studies, we conclude that the opposite regulation of CYP1A1 and CYP1B1 in ER$^-$ versus ER$^+$ cells, is not due directly to ER status *per se*, but likely due to cell type specific changes in levels of protein expression (ie. AhR) and regulatory reprogramming of the cells which accompanies the loss of ER.

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Table 1. Generation of DMBA Metabolites from Microsomal Proteins

Sample	Dihydrodiols				Phenols		Total
	5,6-	8,9-	10,11-	3,4-	A	B	
T47D+							
DMSO	6.9	12	2.6	2.3	22	1.1	46.9
	(14.7)	(25.6)	(5.5)	(4.9)	(46.9)	(2.3)	
TCDD	87	347	30	31	19	4.0	518.0
	(16.8)	(67.0)	(5.8)	(6.0)	(3.7)	(0.8)	
T47D-							
DMSO	9.8	19	3.7	3.6	34	1.3	71.4
	(13.7)	(26.6)	(5.2)	(5.0)	(47.6)	(1.8)	
TCDD	89	325	34	31	20	6.5	505.5
	(17.6)	(64.3)	(6.7)	(6.1)	(4.0)	(1.3)	
MDA+							
DMSO	0.2	3.0[a]		0.0	0.7	1.6	5.5
	(3.6)	(54.5)		(0.0)	(12.7)	(29.1)	
TCDD	4.3	18	2.4	2.1	12	1.9	40.7
	(10.6)	(44.2)	(5.9)	(5.2)	(29.5)	(4.7)	
MDA-							
DMSO	0.8	5.0	0.2	0.5	4.2	1.4	12.1
	(6.6)	(41.3)	(1.7)	(4.1)	(34.7)	(11.6)	
TCDD	6.5	13.8	3.1	2.6	21	2.3	49.3
	(13.2)	(28.0)	(6.3)	(5.3)	(42.6)	(4.7)	

Recombinant Proteins							
rhCYP1A1	189	870	73	79	450	45	1707
	(11.1)	(51.0)	(4.3)	(4.6)	(26.4)	(2.6)	
rhCYP1B1	50	33	27	17	154	2.4	283
	(17.7)	(11.7)	(9.5)	(6.0)	(54.4)	(0.9)	

Microsomes (0.2-1.0 mg), isolated from cells treated for 24 hours with or without 10^{-8} M TCDD, were incubated with 1.5 μM DMBA for 15 min at 37°C, extracted, and analyzed by HPLC for metabolites as described in Materials and Methods. Results were calculated as pmol/mg protein/hr. Values in smaller font in parenthesis are percent of Total metabolites. [a] 8,9- + 10,11-dihydrodiol; at the limit of detection under conditions of analysis, only a single peak observed.

Table 2. Antibody Inhibition of DMBA Metabolism

Sample	Dihydrodiols				Phenols		Total
	5,6-	8,9-	10,11-	3,4-	A	B	
T47D+							
DMSO							
preimmune	6.9	12	2.6	2.3	22	1.1	46.9
	(14.7)	(25.6)	(5.5)	(4.9)	(46.9)	(2.3)	
anti-CYP1A1	7.9	13	3.1	2.7	26	1.1	53.8
	(14.7)	(24.2)	(5.8)	(5.0)	(48.3)	(2.0)	
anti-CYP1B1	0.9	5.0[a]		0.3	4.2	1.4	9.6
	(7.6)	(42.4)		(2.5)	(35.6)	(11.9)	
△ *CYP1B1*	6.0	7.0	2.6	2.0	17.8	0.0	11.8
TCDD							
preimmune	87	347	30	31	19	4.0	518.0
	(16.8)	(67.0)	(5.8)	(6.0)	(3.7)	(0.8)	
anti-CYP1A1	41	99	12	14	92	2.2	260.2
	(15.8)	(38.0)	(4.6)	(5.4)	(35.4)	(0.8)	

Microsomes (0.2-1.0 mg), isolated from T47D+ cells treated for 24 hours with or without 10^{-8} M TCDD, were preincubated for 40 minutes at room temperature with or without anti-CYP antibodies, then incubated with 1.5 µM DMBA for 15 min at 37°C, extracted, and analyzed by HPLC for metabolites as described in Materials and Methods. Results were calculated as pmol/mg protein/hr. Values in smaller font in parenthesis are percent of Total metabolites. [a] 8,9- + 10,11-dihydrodiol; at the limit of detection under conditions of analysis, only a single peak observed.

Table 3. Specific Content and Turnover of CYP1A1 and CYP1B1 in T47D and MDA Cells

	CYP1A1 Specific Content (pmol/mg cellular protein)	CYP1B1 Specific Content (pmol/mg cellular protein)	CYP1A1 Turnover (P450/hr)	CYP1B1 Turnover (P450/hr)
T47D$^+$				
DMSO	n.d.	1.9	n.d.	18.5
TCDD	15.1	35.6	17.2	7.3
T47D$^-$				
DMSO	n.d.	8.6	n.d.	6.2
TCDD	2.3	15.5	112.3	16.3
MDA$^+$				
DMSO	n.d.	0.7	n.d.	5.9
TCDD	3.6	5.0	5.7	4.1
MDA$^-$				
DMSO	n.d.	1.2	n.d.	7.6
TCDD	0.06	12.9	n.d.[a]	1.9

[a] Levels of CYP1A1 protein so low that turnover values could not be calculated.

The pmoles of CYP1A1 and CYP1B1 for determination of specific content were determined by regression analysis against a standard curve of recombinant protein values as described in Methods, and divided by the mg protein in the sample. Specific activity was determined by dividing the total DMBA metabolizing activity (Table 1) adjusted by the percent contribution of the enzyme as determined by antibody inhibition (Table 2) by the specific content.

Table 4. Comparison of Treatments With/Without ICI 182,780

		+TCDD
	+ICI/-ICI	+ICI/-ICI
T47D+		
CYP1A1		
mRNA[a]	nd	2.9
Protein	nd	1.1
CYP1B1		
mRNA[a]	1.4	1.7
Protein	0.8	1.1
MDA+		
CYP1A1		
mRNA[a]	nd	1.4
Protein	nd	0.8
CYP1B1		
mRNA[a]	2.2	1.5
Protein	0.3	1.8

[a] Normalized to ß-actin content.

nd Not Detected

Figure 1. Expression of CYP1A1 and CYP1B1 mRNA in T47D and MDA cells treated with or without TCDD. 10 μg polyA$^+$ RNA from T47D or MDA cells treated with or without 10^{-8} M TCDD for 24 hours was Northern blotted and probed for CYP1A1 or CYP1B1 as described in Materials and Methods. Membrane was exposed to film for 48 hours. Bar graphs represent relative levels of CYP1A1 or CYP1B1 message found in 10 μg polyA$^+$ RNA as determined by densitometry. D = DMSO treated, T = TCDD treated. Ordinate measured in undefined units. Actin standardization was consistent (< 10% variation) within a cell type, however MDA cells contained only about 70% of T47D actin levels (data not shown)

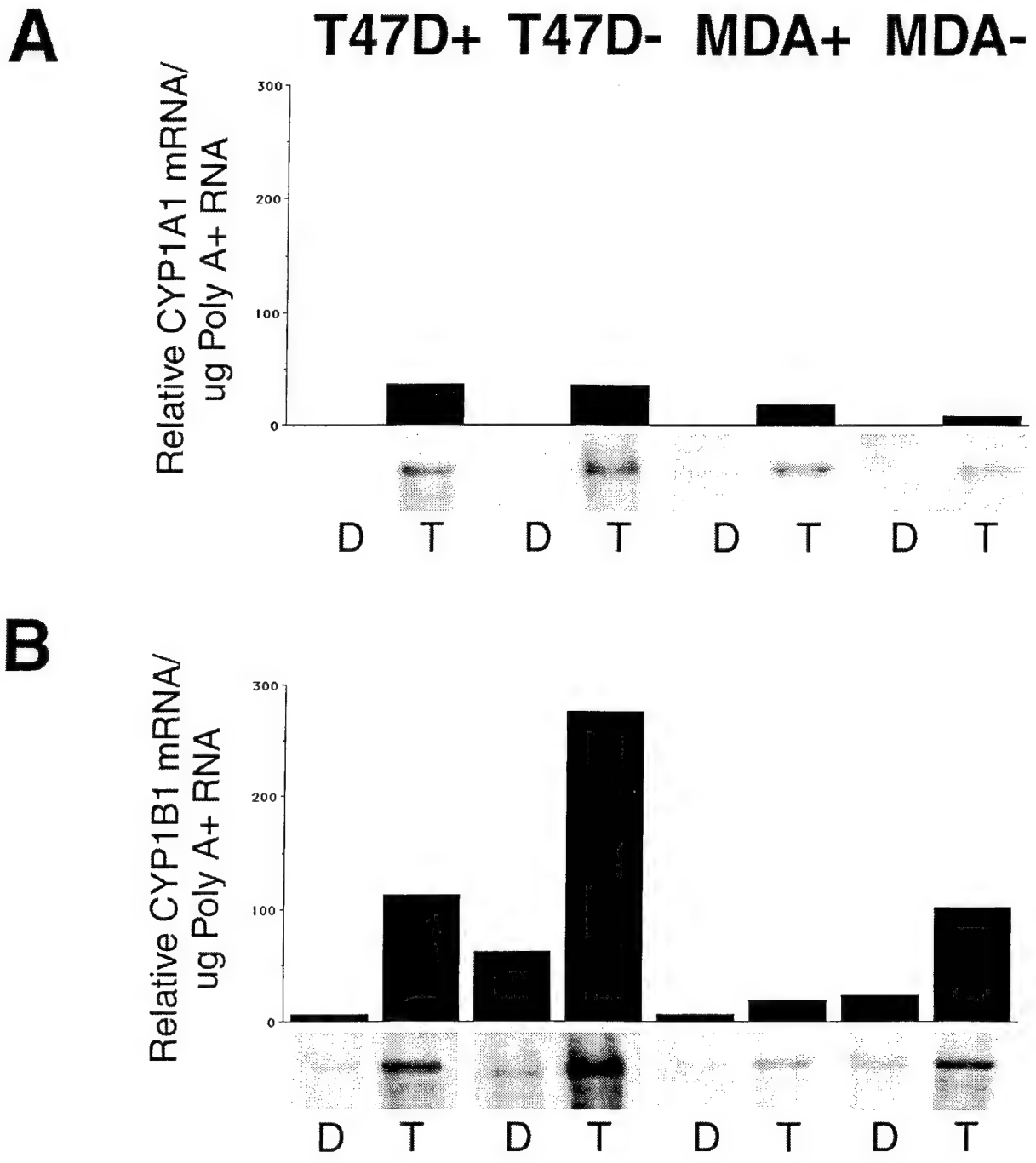
Figure 2. Determination of relative CYP1A1 and CYP1B1 protein levels in T47D and MDA cells treated with or without TCDD. Protein (20 μg) from solubilized T47D or MDA cells treated with or without 10^{-9} M TCDD for 24 hours was Western blotted and probed for CYP1A1 or CYP1B1 as described in Materials and Methods. Bar graphs represent relative levels of CYP1A1 or CYP1B1 protein found in 20 μg total cellular protein as determined by densitometry. D = DMSO treated, T = TCDD treated. Ordinate measured in undefined units.

Figure 3. Determination of estrogen receptor content in T47D and MDA cell clones. ER$^+$ cells were treated with or without 10^{-7} M ICI for 24 hours, followed by treatment with or without 10^{-9} M TCDD for an additional 24 hours in the continued presence/absence of ICI. ER$^-$ cells were treated for TCDD only. 20 μg protein from whole cell solubilizations was immunoblotted for ER as described in Materials and Methods. D = DMSO treated, T = TCDD treated.

Figure 4. Effect of ICI 182,780 on CYP1A1 and CYP1B1 expression in T47D$^+$ and MDA$^+$ cells. Cells were treated with or without 10^{-7} M ICI for 24 hours, followed

by treatment with or without 10^{-9} M TCDD for an additional 24 hours in the continued presence/absence of ICI. (A) Northern blot analysis of 10 μg poly A^+ RNA probed with CYP1A1 or (B) CYP1B1, as described in Materials and Methods. Bar graphs represent relative levels of message expression as determined by densitometry. D = DMSO treated, T = TCDD treated. Ordinates measured in undefined units. Actin standardization was consistent (< 10% variation) within a cell type, however MDA cells contained only about 70% of T47D actin levels.

Figure 5. Determination of Ah receptor and ARNT content in T47D and MDA cell clones. Cells were treated with or without 10^{-9} M TCDD for 24 hours and protein from whole cell solubilizations was immunoblotted for AhR (20 μg) or ARNT (μg) as described in Materials and Methods. D = DMSO treated, T = TCDD treated.


A
T47D+ T47D- MDA+ MDA-
Relative CYP1A1 mRNA/
ug Poly A+ RNA
300
200
100
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D T D T D T D T
B
Relative CYP1B1 mRNA/
ug Poly A+ RNA
300
200
100
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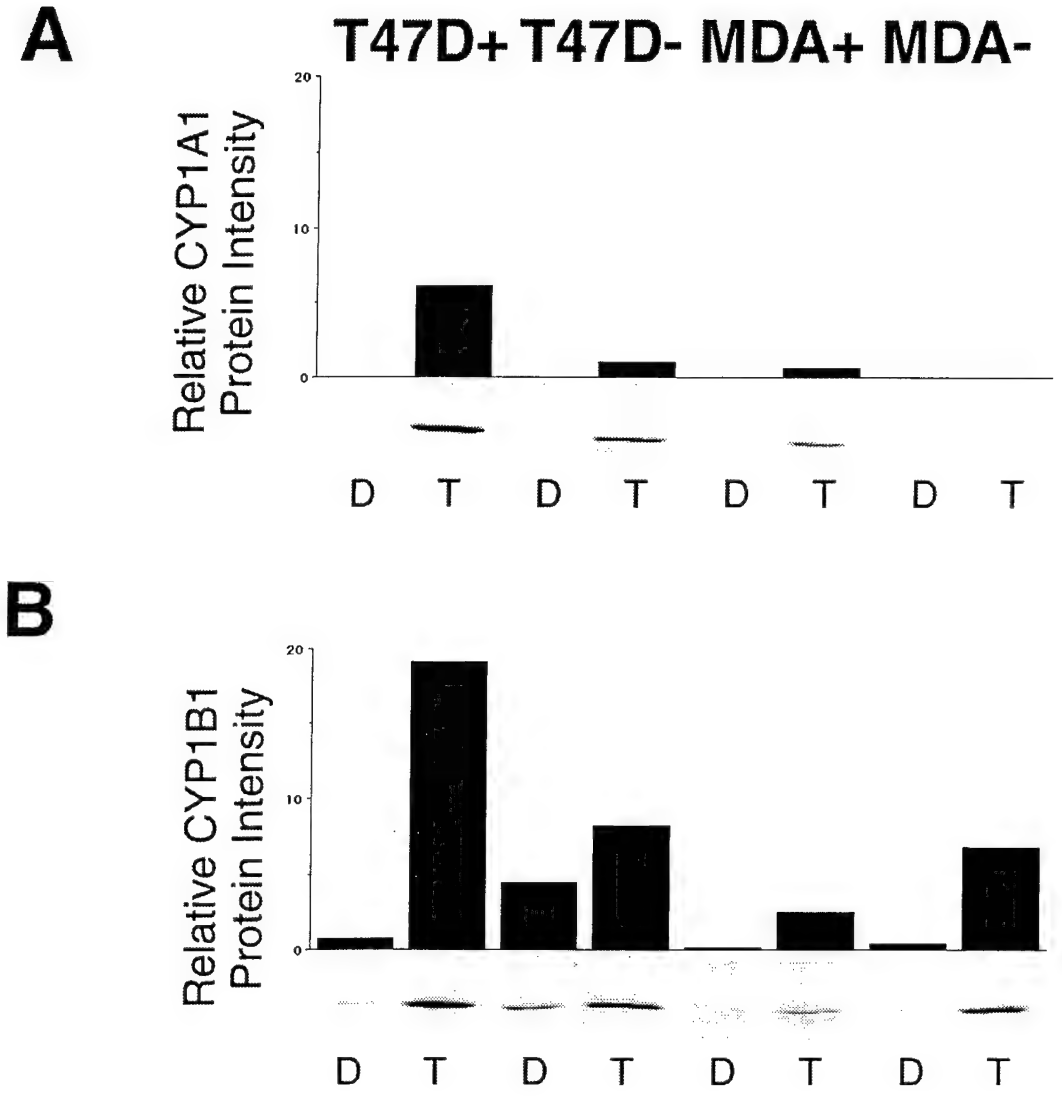
Figure 1


A
T47D+ T47D- MDA+ MDA-
Relative CYP1A1 Protein Intensity
20
10
0
D T D T D T D T
B
Relative CYP1B1 Protein Intensity
20
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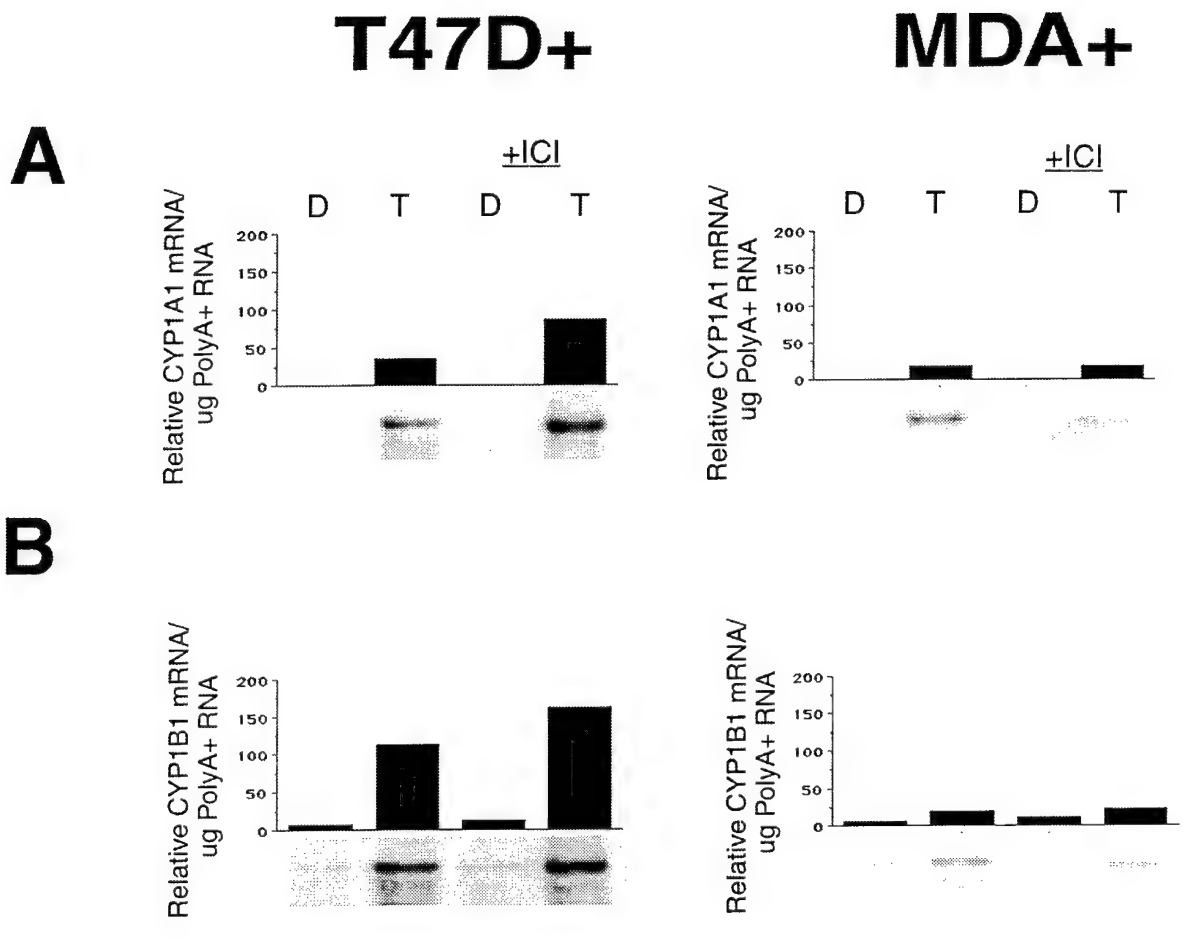
D T D T D T D T

Figure 2.

Figure 3


T47D+
MDA+
A
+ICI
D T D T
Relative CYP1A1 mRNA/ ug PolyA+ RNA
200
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Relative CYP1B1 mRNA/ ug PolyA+ RNA

Figure 4

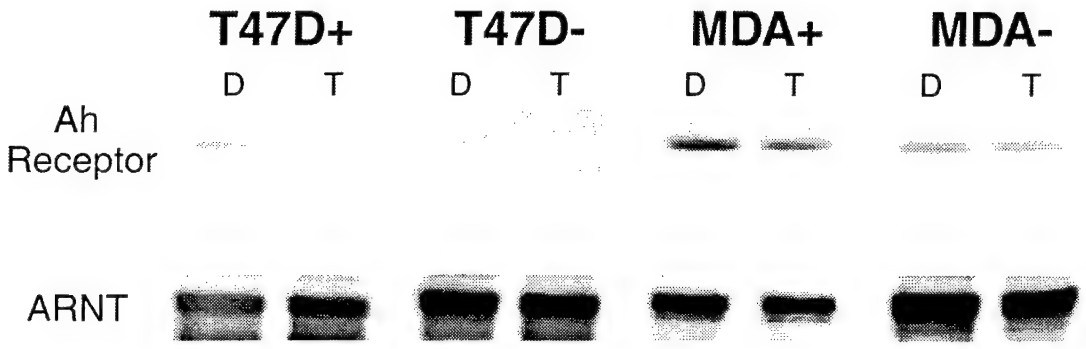

Figure 5

Characterization of CYP1B1 and CYP1A1 Expression in Human Mammary Epithelial Cells: Role of the Aryl Hydrocarbon Receptor in Polycyclic Aromatic Hydrocarbon Metabolism

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Running Title: CYP1B1 and CYP1A1 Expression in Human Mammary Epithelial Cells.

Key Words: CYP1B1, CYP1A1, polycyclic aromatic hydrocarbon, human mammary epithelial cell, aryl hydrocarbon receptor.

Footnotes:

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[II] This research was supported by NIEHS grant 144EN46 and DOD Breast Cancer Research Grant DAMD17-94-J-4054.

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[IV] Abbreviations: PAH, polycyclic aromatic hydrocarbon; CYP, cytochrome P450; DMBA, 7,12-dimethylbenz(a)anthracene; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; Ah receptor, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon nuclear translocator protein; HMEC, human mammary epithelial cell; PCR, polymerase chain reaction; RMEC, rat mammary epithelial cells; ECL, enhanced chemiluminescence.

[VI] Acknowledgment: We would like the thank Dr. Michael Gould and Wendy Kennan (University of Wisconsin Clinical Cancer Center) for generously providing the HMEC organoid preparations utilized in this study.

ABSTRACT

CYP1B1 and CYP1A1 expression and metabolism of 7,12-dimethylbenz(a)anthracene (DMBA) has been characterized in early passage human mammary epithelial cells (HMEC) isolated from reduction mammoplasty tissue of eight individual donors. The level of constitutive microsomal CYP1B1 protein expression was donor-dependent (<0.01- to 1.4-pmol/mg microsomal protein). CYP1B1 expression was substantially induced by exposure of the cells to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) to levels ranging from 2.3- to 16.6-pmol/mg among the eight individual donors. Extremely low, reproducible levels of constitutive CYP1A1 expression were detectable in three donors (0.03- to 0.16-pmol/mg microsomal protein). TCDD inductions were larger for CYP1A1, as compared to CYP1B1, demonstrating substantial variability in the induced levels among the donors (0.8- to 16.5-pmol/mg). Northern and rtPCR analyses corroborated that these donor dependent differences were transcriptionally regulated. CYP1B1 mRNA (5.2 kb) was constitutively expressed and was highly induced by TCDD, consistent with immunodetectable protein expression. Cytochrome P450-mediated DMBA metabolism, in secondary cultures, was maximum at day 6 and subsequently decreased concomitant with an increase in the proportion of basal relative to luminal HMEC. The contributions of CYP1B1 and CYP1A1 to the metabolism of DMBA were analyzed utilizing recombinant human CYP1B1 and CYP1A1, as references, in conjunction with antibody-specific inhibition analyses (anti-CYP1B1 and anti-CYP1A1). Constitutive microsomal activity exhibited a profile of regioselective DMBA metabolism that was characteristic of human CYP1B1 (increased proportions of 5,6- and 10,11-DMBA-dihydrodiols) which was inhibited by anti-CYP1B1 (84%), but not by anti-CYP1A1. TCDD-induced HMEC microsomal DMBA metabolism generated the 8,9-dihydrodiol of DMBA as the predominant metabolite with a regioselectivity similar to that of recombinant human CYP1A1, and which was subsequently inhibited by anti-CYP1A1 (79%). A CYP1B1 contribution was indicated by the regioselectivity of residual metabolism and by anti-CYP1B1 inhibition (25%). DMBA metabolism analyses of one of three donors expressing measurable basal expression of CYP1A1 confirmed DMBA metabolism levels equivalent to that from CYP1B1. The HMEC of all donors expressed similar, very high levels of the aryl hydrocarbon receptor (AhR) and the aryl hydrocarbon nuclear translocator protein (Arnt), suggesting that AhR and Arnt expression are

not responsible for differences in cytochrome P450 expression. This study indicates that CYP1B1 is an important activator of polycyclic aromatic hydrocarbons (PAH's) in the mammary gland when environmental chemical exposures minimally induce CYP1A1. Additionally, certain individuals express low levels of basal CYP1A1 in HMEC, representing a potential risk factor of mammary carcinogenesis through enhanced PAH bioactivation.

INTRODUCTION

In the United States, breast cancer represents the second leading cause of cancer-related death in women (1). The etiology of breast cancer remains unknown, although developmental, genetic, endocrine, dietary, and environmental factors have been implicated as risk factors for mammary tumorigenesis. Environmental contaminants, including polycyclic aromatic hydrocarbons (PAH's), have been associated with chemical-mediated carcinogenesis in rodent models. In humans, mammary tissue is an important target site for PAH-mediated carcinogenesis, particularly since PAH's partition into mammary adipose tissue (2-8).

The cytochrome P450 superfamily of drug metabolizing enzymes mediates the detoxication of environmental contaminants, including PAH's (9-11). However, PAH's often undergo bioactivation, thereby acquiring carcinogenic and mutagenic potential as a consequence of the oxidative metabolic process (12-14). Mutagenesis analyses have shown that rat mammary epithelial cells (RMEC) and human mammary epithelial cells (HMEC) preferentially activate 7,12-dimethylbenz(a)anthracene (DMBA) and benzo(a)pyrene (B(a)P), respectively, to mutagenic dihydrodiol epoxides, which are the most potent cancer initiators produced by these compounds (15). The bioactivated PAH's have been implicated in the development of a variety of rodent tumors, including mammary cancer, by inducing mutations in genes that control cell proliferation (H-ras) or regulate cellular adaptation to chemical-mediated damage (p53) (16-19). In addition, cH-ras gene amplification has been demonstrated in primary HMEC following treatment with activated chemical carcinogens, and the immortalization and transformation of HMEC has been observed following treatment with PAH's (20-23).

The present study focuses on the CYP1 superfamily of cytochrome P450 isozymes which are largely responsible for both PAH activation and detoxication in many cells (24-25). The CYP1A family of P450 cytochromes is composed of CYP1A1 which is broadly expressed after PAH induction, but rarely observed constitutively, and CYP1A2 which is constitutively expressed and TCDD-inducible almost exclusively in the liver (26). PAH-mediated induction of CYP1A1 and CYP1A2 is stimulated by ligand binding to the aryl hydrocarbon receptor (AhR) (24). TCDD, the most potent agonist of the AhR, mediates the transcriptional activation of CYP1A1 via a cellular cascade initiated by ligand binding, followed by dissociation of the 90 kDa heat shock protein, hsp 90, nuclear translocation and association with the aryl hydrocarbon nuclear translocator (Arnt) protein and nuclear dioxin responsive elements (DRE). The transcriptional activation of human CYP1A1 is highly cell-line specific in mammary carcinoma-derived cell lines (27-28).

This laboratory has recently purified and cloned an additional PAH-responsive cytochrome P450 of the CYP1 gene family, CYP1B1, from C3H10T1/2 mouse embryo fibroblasts and rat

adrenal cells (29-30). The human ortholog has been cloned from a human squamous carcinoma cell line, SCC-13 (31-32). CYP1B1 appears to be preferentially localized in tissues of mesodermal origin, particularly those producing steroids (adrenal, testis, and ovary) or those responding to steroids (prostate, uterus, and mammary tissues) (29-30, 33). Interestingly, a deficiency in human CYP1B1 protein expression has recently been shown to be responsible for the development of congenital glaucoma (34). CYP1B1 is expressed constitutively, and is TCDD-inducible, in the human mammary carcinoma MCF-7 cell line (35). Human CYP1B1 has been associated with the hydroxylation of 17β-estradiol to form the 4-catecholestrogen in analyses utilizing recombinantly expressed human CYP1B1, as well as in the MCF-7 cell line (36-37). This activity has been shown to be enhanced in human mammary and uterine tumors relative to the surrounding tissue (38-40). Collectively, these findings clearly establish a developmental, as well as an endogenous, role for this P450 cytochrome in estrogen metabolism, in addition to a role in PAH bioactivation.

Mutagenesis analyses have recently demonstrated that human CYP1B1 activates numerous structurally diverse environmental contaminants, including PAH's and their dihydrodiol derivatives, heterocyclic and aryl amines, and nitroaromatic hydrocarbons, to genotoxic agents (33). The selectivity of these CYP1B1-specific responses differs substantially from those mediated by CYP1A1. Thus, the CYP1B1-mediated activation of many environmentally persistent xenobiotics likely plays a significant role in chemical-induced mammary carcinogenesis. Although previous studies have demonstrated species-related differences in PAH metabolism and bioactivation with respect to rat and human mammary epithelial cells, these studies subjected the cells to prolonged exposures of PAH (24 to 42 hours) and, thus, are representative of inductive as well as activation processes (15, 41-42). Subsequent analyses have shown that PAH metabolism in cultured rat mammary cells is mediated by both CYP1A1 and CYP1B1, in a cell selective manner (43). In rat mammary fibroblasts (RMF), CYP1B1 was shown to be exclusively expressed both constitutively and following TCDD-induction. However, in RMEC CYP1A1, absent constitutively, was highly inducible by TCDD, while CYP1B1 was scarcely detectable, even under conditions of TCDD exposure. In spite of these differences in cell culture, immunohistochemical analyses have recently shown that CYP1B1 is extensively expressed in rat mammary ductal epithelia, demonstrating that CYP1B1 is, indeed, expressed in mammary epithelial cells *in vivo* (44). While CYP1A1 and CYP1B1 are each expressed in human mammary tumor cell lines, little is known regarding CYP1B1 and CYP1A1 cell type-specific expression in cultured human mammary cells. This is of particular importance since many studies have emphasized the multiplicity of cell types existent in HMEC cultures, and the dependence of culture conditions on cell specific expression (45).

The present study characterizes the expression of CYP1A1 and CYP1B1, the major PAH-metabolizing P450 cytochromes, in early passage HMEC. The cells have been cultured under

conditions utilized in previous PAH-induction studies (15, 41-42), and are composed of a heterogeneous population of basal and luminal epithelial cells. This study represents the first analysis of functional cytochrome P450 expression in cultured HMEC employing conditions that measure basal levels of expression and activity, distinct from the induction response. HMEC isolated from eight women (5 younger, 17-22 years of age; 3 older, 35-49 years of age) following reduction mammoplasty surgeries were utilized in this study. We have quantitated constitutive and TCDD-inducible expression of CYP1B1 and CYP1A1 with respect to the level of AhR and Arnt expression, two proteins which may influence the extent of PAH-inducible cytochrome P450 expression. DMBA metabolism was utilized as a biomarker for functional CYP1B1 and CYP1A1 expression. The regioselective profiles of recombinant human CYP1B1- and CYP1A1-mediated DMBA metabolism, in combination with antibody inhibition analyses, have been utilized to resolve the respective contributions of these P450 cytochromes to both cellular and microsomal metabolism. This study provides evidence that the relative contributions of CYP1B1 and CYP1A1 to cellular bioactivation are highly dependent upon the magnitude of the cellular exposure to environmental chemicals (e.g. PAH's). We provide evidence that CYP1B1 is an important contributor to PAH bioactivation under conditions of low induction from environmental exposure. Additionally, we show that there are substantial differences in CYP1B1 and CYP1A1 expression that may represent a risk factor for chemical-mediated breast cancer.

MATERIALS AND METHODS

Materials:

Mammary epithelial growth media (MEGM-bullet kit) was purchased from Clonetics, Inc. (San Diego, CA.). TRIzol reagent was obtained from GibcoBRL (Grand Island, NY.). Random 9-mers and the random-primed DNA labeling kit were obtained from Stratagene (La Jolla, CA.). The horseradish peroxidase-conjugated goat anti-rabbit IgG, Taq DNA polymerase, reverse transcriptase, dNTP's and RNasin were purchased from Promega (Madison, WI). PCR primers were obtained from Integrated DNA Technologies (Iowa City, IA) The enhanced chemiluminescence (ECL) detection system was purchased from Amersham (Arlington Heights, IL.) The BCA protein assay reagent was obtained from Pierce (Rockford, IL.). Nitrocellulose was supplied by Schleicher and Schuell (Keene, NH), while all other materials used for SDS-PAGE were obtained from Bio-Rad Laboratories (Richmond CA.). DMBA was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). HPLC grade methanol was purchased from VWR Scientific (Detroit, MI). Human recombinant CYP1B1 and CYP1A1 microsomes and human

epoxide hydratase were obtained from Gentest Corporation (Woburn, MA). All other chemicals and reagents were purchased from Sigma Chemical Co. and were of the highest quality available.

Primary Antibodies and Epoxide Hydratase:

Polyclonal antiserum (IgG fraction) specifically recognizing CYP1A1 was generated in this laboratory, as previously described (46). Anti-peptide antiserum specifically recognizing human CYP1B1 was provided by Dr. Craig Marcus (University of New Mexico) and Dr. William Greenlee (University of Massachusetts) (47). Monoclonal antibodies specific for the Ah-receptor was provided by Dr. Gary Perdew (Penn State University), while polyclonal antisera specifically recognizing Arnt was a gift from Dr. Richard Pollenz (University of South Carolina) (48). Polyclonal antibodies to mouse CYP1B1 (IgY) were prepared in this laboratory in female Leghorn chickens and purified from the eggs, as previously described (49).

Cells and Cell Culture:

Human mammary organoid preparations isolated from eight individual donors (Table 1) were provided by Dr. Michael Gould and the UW Comprehensive Cancer Center Cell Bank. The mammary tissue was processed and organoids isolated in the Gould laboratory, according to previously published procedures (4,15). Primary organoids were plated ($2x10^4$ cells/cm^2) on plastic in mammary epithelial growth media (MEGM) supplemented with penicillin-streptomycin (50 U/ml penicillin, 0.05 mg/ml streptomycin). Cells were grown at 37°C in a humidified environment of 5% CO_2 and 95% air. Confluent cells (7-10 days post-plating) were passaged by differential trypsinization, plated at $1.7x10^4$ cells/cm^2, and subsequently analyzed at day 4, 6, or 13.

Cells either remained untreated or were treated with TCDD (10 nM) for a period of 24 hours prior to analysis. Cells utilized for microsomal preparations were harvested by trypsinization and washed three times with ice cold 1X PBS. Microsomal fractions were isolated by differential centrifugation, as previously described (46). Protein concentrations were determined according to the bicinchoninic acid procedure using the Pierce BCA protein assay kit, according to manufacturer's protocol, using bovine serum albumin as the standard.

DMBA Metabolism Assay:

Microsomal DMBA metabolism was analyzed as previously described (44). Briefly, microsomal incubations were composed of 0.2 and 1.0 mg/ml microsomal protein for induced and basal metabolism, respectively, 14.2 mM glucose 6-phosphate, 0.06 U glucose 6 phosphate dehydrogenase, 1.2 mM NADP, 6 mM $MgCl_2$, and 0.1 M potassium phosphate (pH 7.6) to a total reaction volume of 1 ml. The reaction was initiated by the addition of 1.5 μM DMBA (HPLC

purified) and allowed to incubate for 15 min at 37°C, under conditions of subdued lighting. The analysis of the human recombinant CYP1B1 and CYP1A1 microsomal standards, 0.075- and 0.050 mg microsomal protein/ml, respectively, were assayed, as above, with the addition of 0.5 mg/ml human epoxide hydratase. Metabolites were extracted and the microsomal DMBA metabolic profiles were examined by reverse phase HPLC analysis. All data has been normalized relative to DMBA recovery following extraction procedures.

Cellular DMBA metabolism was examined in 12-well culture plates (3.8 cm^2/well) on actively growing cells. Each incubation was composed of 0.5 ml of culture media supplemented with 10 μM DMBA. Basal and TCDD-induced cellular incubations were completed at 37°C for 60 and 30 min., respectively. The incubation medium was removed from the culture vessel and was incubated with β–glucuronidase (1000 U in 0.5 M NaOAc, pH 5.2) for 4 h at 37°C to effect the release of all H_20-soluble metabolites. DMBA metabolites were extracted and HPLC metabolic profiles examined, as described above.

Antibody Inhibition Analysis:

Antibody inhibition of microsomal DMBA metabolism was analyzed by the addition of anti-CYP1B1 (chicken; IgY, 5 mg Ab/mg microsomal protein) and anti-CYP1A1 (rabbit; IgG, 10 mg Ab/mg microsomal protein), concentrations previously determined to optimally inhibit CYP1B1- and CYP1A1-specific activities in constitutive and TCDD-induced MCF-7 cells. The corresponding pre-immune IgY or IgG was added at equivalent concentrations to serve as controls. All reaction mixtures were incubated for 45 min at room temperature prior to assaying DMBA metabolism (35).

Immunoblot Analysis:

Microsomal proteins were subjected to SDS-PAGE (3% acrylamide stacking gel, 7.5% acrylamide separating gel) followed by immunoblot analysis, as previously described (50-51). Immunoreactive proteins were visualized using the ECL method of detection, according to the manufacturer's protocol. Cytochrome P450 expression was quantitated relative to human recombinant CYP1B1 (74 pmol P450/mg microsomal protein) and CYP1A1 (104 pmol P450/mg microsomal protein) microsomal standards. Quantitation was completed by soft laser scanning densitometry employing a Zeineh Model SL-504-XL soft laser scanning densitometer (Fullerton, CA).

Measurement of mRNA by rt-PCR:

Total RNA was prepared from day 6 HMEC cultures which were either untreated or treated with 10 nM TCDD for 6 h prior to isolation. Cells were harvested with TRIzol reagent and the

RNA isolated according to the manufacturer's protocol. cDNA templates for PCR were synthesized as follows: total RNA (2 μg) and the random 9-mer primer mix (1 μg), in a total volume of 31 μl, were heated to 60°C for 5 min. The samples were allowed to cool slowly to room temperature followed by the addition of the reverse transcription master mix composed of 1.0 mM of each dNTP, 40 U RNasin, 10.0 mM DTT, and 200 U M-MLV reverse transcriptase, to total of 50μl. The samples were incubated at 40°C for 1 h followed by denaturation for 5 min at 100°C.

Primers specific to CYP1B1 (fp: 5'-CGTACCGGCCACTATCACTG-3'; rp: 5'-GCAGGCTCATTTGGGTTGGC-3') and CYP1A1 (fp: 5'-AAGCACGTTGCAGGAGCTGATG-3'; rp: ACATTGGCGTTCTCATCCAGCTGCT-3') were synthesized using an Applied Biosystems 380A synthesized. All PCR amplifications were completed in a Perkin Elmer Cetus model 480 DNA thermocycler. CYP1B1 amplifications were composed of 2 μg cDNA template, 0.22 mM of each dNTP, 0.625 U Taq DNA polymerase, and 0.56 μM of each primer, and H_2O to a final volume of 22.5 μl. The reaction was initiated under "hot start" conditions whereby, the samples were heated to 94°C for 3 min followed by the addition of 2.78 mM $MgCl_2$. The samples were cycled for 1 min at 94°C, 1.5 min at 60°C, and 2.0 min at 72°C for a total of 30 cycles. CYP1A1 amplifications were composed of 2 μg cDNA template, 0.6 mM of each dNTP, 2.5 mM $MgCl_2$, 0.625 U Taq DNA polymerase, and 0.5 μM of each PCR primer and H_2O to a total volume of 25 μl. Cycle parameters were 1 min at 94°C, 1.5 min at 57°C, and 2.0 min at 72°C for a total of 30 cycles. PCR products were analyzed by agarose gel (1.6% agarose) chromatography and visualized by staining in ethidium bromide.

Northern Analysis:

cDNA probes specific for CYP1B1 and β-actin were labeled with [α-^{32}P]dCTP (400 Ci/mmol) using the random-primed DNA labeling kit, as per manufacturer's protocol. The poly(A)$^+$ mRNA was isolated from day 6 HMEC cultures according to the method of Badley *et al* (52). mRNA was fractionated in 1.2% agarose / 37% formaldehyde gels, transferred to nitrocellulose, and probed as previously described (53).

RESULTS

Human mammary epithelial cells were cultured from eight individual donors. Organoid preparations, isolated from reduction mammoplasty procedures and cultured under serum-free conditions, generally demonstrated full attachment within 3- to 4- days followed by a period of rapid proliferative growth, reaching confluence within 7-10 days of initial plating. The proliferative efficiency varied among cultures of the eight individual donors, with donors A and C

demonstrating the slowest rate of growth. Morphologically, the heterogeneity of the cultures was readily apparent, as shown in Fig. 1. The cultures were composed of at least two distinct cellular subtypes, as characterized by dense areas of tightly packed cells encircled by uniform margins (Fig. 1A) and regions displaying the cobble-stone appearance typified by cultured mammary epithelial cells (Fig. 1B). These cultures demonstrated areas of proliferative growth of each distinct subtype, as well as areas of collateral growth (Fig. 1C). The cultures depicted in Figure 1 are representative of all donors examined in this study. Kao *et al,* have recently reported similar observations under comparable culture conditions, and they have immunocytochemically characterized the cells, using antisera specific to the cytokeratin 18 and cytokeratin 14 cell markers, as representing luminal (Fig 1A) and basal (Fig 1B) epithelial subtypes, respectively (54).

Due to a limited availability of organoids from several of the donors utilized in this study, not all experimental analyses have been completed on cultures of each individual. Table 1 provides a summary of the analyses that have been completed on each individual donor. These eight donors divide into 5 young donors (A-E; age 17-22) and 3 older donors (F-H; age 35-40).

Cellular DMBA metabolism analysis demonstrated optimal enzymatic activity at day 6 of culture. DMBA metabolism was examined in secondary HMEC cultures in order to evaluate the time dependence of functional cytochrome P450 expression. Cellular DMBA metabolism of three donors was analyzed at days 4, 6, and 13 of culture. Figure 2 depicts the cellular metabolism of one donor, donor D, but the data is representative of all three individuals examined. HMEC were exposed to 10 nM TCDD for a period of 24 h prior to analysis. The level of TCDD-induced cellular metabolism increased until day 6 of culture followed by a greater than 60% decline in activity between day 6 and day 13. Thus, all subsequent experiments were completed on cells harvested at day 6 of culture to representing optimal expression of functional P450 cytochromes.

TCDD-induced DMBA metabolism was examined in the intact cell (cellular assay), as well as in the isolated microsomal and nuclear/mitochondrial fractions. As shown in Table 2, similar regioselective profiles of DMBA-dihydrodiol formation were observed in each of the three assay systems. The rate of TCDD-induced metabolism in the intact cells was of a similar magnitude as that obtained in the microsomal assay (calculating 1 mg microsomal protein/10^7 cells). However, basal cellular metabolism was not measurable at these cell densities (10^5 cells/well) in assays which were of short enough duration to exclude induction by DMBA, an effective AhR agonist (55). Therefore, we have focused on the analysis of DMBA metabolism in the isolated microsomal fraction of basal and TCDD-induced cells. It is interesting to note, however, that as much as 40% of the DMBA metabolism is potentially associated with P450 cytochromes localized within the

perinuclear endoplasmic reticulum, indicating that functional P450 expression is not restricted to the microsomal fraction of the cell.

CYP1B1 is expressed constitutively while CYP1B1 and CYP1A1 are TCDD-inducible in normal HMEC. Microsomal CYP1B1 and CYP1A1 expression was examined by immunoblot analysis in eight HMEC preparations at day 6 of culture. The expression of CYP1B1 and CYP1A1 in six donor preparations relative to recombinant human microsomal standards is shown in Fig. 3. The level of CYP1B1 and CYP1A1 expression in a seventh individual, donor A, was within the same range as the other donors. The magnitude of expression has been quantitated for CYP1A1 and CYP1B1, each relative to recombinant human standards (Fig. 4). These values represent extremely low levels of expression (<0.3- to 16-pmol/mg), but were reproducibly quantifiable.

Each individual exhibited constitutive CYP1B1 expression which was substantially induced by TCDD. The variability in TCDD-induced levels among the donors was similar to that observed for basal CYP1B1. The average levels of basal and induced CYP1B1 expression among the six individuals were 0.9 (±0.5)- and 10.4 (±4.7)-pmol/mg, respectively. The level of constitutive expression of CYP1A1 was substantially lower than that of CYP1B1. Constitutive microsomal CYP1A1 expression was at the lower limit of detection (≤0.02 pmol/mg) in four of the donors (A, B, C, and D), while an extremely low level of expression (0.03- to 1.6-pmol CYP1A1/mg) was detectable in cultures of the donors E, F, and G (employing exposure conditions 10 times longer than required for CYP1B1 detection). However, the magnitude of TCDD-mediated induction was substantially greater for CYP1A1 (over 100-fold) than for CYP1B1 (5- to 25-fold; excluding donor A), resulting in comparable TCDD-induced microsomal levels of the two P450 cytochromes. TCDD-induced CYP1A1 expression was more variable between the individual donors than induced CYP1B1 expression (varying by 21- and 7-fold, respectively, between donors A and B, donors demonstrating the highest and lowest levels of expression). Thus, TCDD-induced levels of CYP1A1 and CYP1B1 were weakly correlated (r^2=0.56) (Fig. 5).

The analysis of the HMEC isolated from donor H (age 49) was completed on passage 2 cultures, as a limited number of organoids were available and, therefore, more extensive cellular expansion was required in order to obtain a sufficient quantity of cells to complete immunoblotting procedures. Passage 2 cultures from a typical donor (G; age 40) exhibited only slightly reduced constitutive and TCDD-induced expression of CYP1B1, as measured relative to passage 1 cultures (Fig. 6). However, the extended passage substantially decreased basal and TCDD-induced CYP1A1 expression, reducing the level of immunodetectable protein by as much as 5-fold. Passage 2 cultures from donors G and H demonstrated much greater individual differences than were observed between donors in passage 1 cultures. Thus, donor H expressed much lower levels

of basal and induced CYP1B1, as compared to passage 2 cultures of donor G, while demonstrating higher levels of basal and induced CYP1A1 expression.

Microsomal DMBA metabolism parallels immunodetectable cytochrome P450 expression. The analysis of DMBA metabolism mediated by recombinant CYP1B1 and CYP1A1 expressed in lymphocyte microsomes demonstrated isoform-specific differences with respect to the pattern of regioselective product distribution (Table 3). However, the profiles were not as distinct as previously demonstrated with the rodent orthologs (44). Human CYP1A1-mediated DMBA metabolism demonstrated relatively low levels of 5,6- and 10,11-dihydrodiol formation relative to the 8,9- DMBA-dihydrodiol (ratios of 0.22 and 0.08, respectively). Conversely, human CYP1B1-directed metabolism generated equivalent levels of the 5,6- and 10,11-DMBA-dihydrodiols, relative to the 8,9-dihydrodiol of DMBA (ratios of 1.5 and 0.8, respectively). In addition, the rate of DMBA metabolism mediated by human CYP1A1 exceeded that of CYP1B1 by approximately 6-fold.

DMBA metabolism was analyzed as a biomarker of functional cytochrome P450 expression in HMEC at day 6 of culture, in parallel with the previous immunoblot analyses. Table 3 compares the regioselective profile of constitutive and TCDD-induced DMBA metabolism generated by donors D and E. These two donors represent the range of activities observed among all of the donors utilized in this study. The variability in the rate of constitutive metabolism exhibited by donors D and E is consistent with an approximately 2.5-fold difference in the level of constitutive CYP1A1 protein expression. The constitutive microsomal DMBA metabolism of donor D demonstrated equivalent proportions of 5,6- and 10,11- DMBA-dihydrodiol metabolites with low 8,9-dihydrodiol formation. This reproducible pattern of regioselective metabolism is similar, but reproducibly different from the recombinant human CYP1B1. In contrast, the profile of constitutive metabolism generated by donor E clearly demonstrated a higher rate of metabolism reflective of an approximately equal component of CYP1A1-directed activity, as indicated by low ratios of 5,6-/ 8,9- and 10,11-/8,9- metabolite formation. This provides an important confirmation of the detection of constitutive CYP1A1 protein in cells from this donor.

The proportion of the 5,6-, and 10,11-dihydrodiol metabolites, relative to the 8,9-dihydrodiols, generated by the TCDD-induced microsomes of donor D and E were consistent with the predominant involvement of CYP1A1. These profiles are typical of trends demonstrated by all donors following TCDD induction.

Antibody inhibition of DMBA metabolism further defined the contributions of CYP1B1 and CYP1A1 to DMBA metabolism in the HMEC (Table 4). The regioselective distribution of metabolism that is blocked by the specific antibody provides a characteristic profile of the inhibited enzyme. The addition of anti-CYP1A1 did not substantial effect basal activity (7% inhibition),

while a parallel addition of anti-CYP1B1 inhibited DMBA-dihydrodiol formation by 84%. The addition of anti-CYP1A1 to TCDD-induced microsomal fractions reduced dihydrodiol formation by 79%, and left residual activity with a regioselective metabolite distribution consistent with CYP1B1-mediated metabolism. The initial TCDD-induced activity was inhibited by 25% by anti-CYP1B1, while the simultaneous addition of anti-CYP1A1 and anti-CYP1B1 to induced microsomes increased the extent of inhibition from 78- to 91%. This suggests a contribution of approximately 15 to 25% by CYP1B1 to these TCDD-induced activities. The observed equal expression of the proteins combined with a 6-fold higher specific activity for CYP1A1 predicts a 14 percent contribution from CYP1B1. This is fully consistent with the antibody inhibition data within the variability of these assays. Thus, when TCDD stimulates DMBA metabolism by 30- to 40-fold, the 5-fold increase in CYP1B1-mediated activity is overwhelmed by the contribution from the more than 100-fold induction of the more active CYP1A1.

As discussed above, HMEC from donor E consistently exhibited a DMBA product profile characteristic of a mixture of CYP1B1- and CYP1A1-mediated metabolism. This was confirmed by antibody inhibition, whereby CYP1B1 and CYP1A1 each inhibited this metabolism by 35%, again, consistent with equal contributions from each form (Table 5).

The analysis of DMBA metabolism with respect to the measurement of cytochrome P450 protein expression showed that CYP1B1 turnover in HMEC was 6 h^{-1} (constitutive microsomes; Donor D and E), while CYP1A1 turnover was 90 h^{-1} in constitutive microsomes and 21 h^{-1} in TCDD-induced microsomes. The values for recombinant CYP1B1 and CYP1A1 in lymphoblast microsomes were 4- and 17 h^{-1}, respectively.

rtPCR and Northern blot analyses of RNA expression of CYP1B1 and CYP1A1 in HMEC supports immunoblot analysis. Constitutive and TCDD-inducible CYP1B1 and CYP1A1 expression were analyzed utilizing total RNA isolated from cultured HMEC of three donors (A, B, and D), by rtPCR methodologies (Fig. 7). Constitutive and TCDD-inducible CYP1B1 mRNA expression was detectable in each of the donors examined. The approximate 10-fold increase CYP1B1 mRNA was fully consistent with the induction of immunodetectable CYP1B1 protein following TCDD exposure (Fig. 4). Constitutive CYP1A1 mRNA expression was barely detectable in cells from these donors but this, again, parallels the near absence of protein expression. We have yet to examine mRNA from donors E, F, and G which express detectable constitutive CYP1A1 protein. However, in a manner paralleling protein expression, TCDD-inducible CYP1A1 was observed in message isolated from donors A, B, and D. Furthermore, the lower level of TCDD-induced CYP1A1 mRNA observed from cultures of donor A corroborate immunodetectable protein expression. Interestingly, cells from this donor, like those from donor C, grew very slowly in culture.

Northern blot analysis confirmed the expression of constitutive CYP1B1 mRNA which was highly inducible by TCDD (5- to 30-fold) (Fig. 8). The magnitude of CYP1B1 induction, in response to TCDD, was greater in the cultured HMEC than in an established mammary tumor cell line (MCF-7). The level of message expression was highly variable among the donors, confirming that the donor dependent differences demonstrated in the immunoblot and rtPCR analyses are the result of transcriptional regulation.

Ah-receptor and Arnt expression is elevated in cultured HMEC. Cytosolic Ah-receptor (AhR) expression was analyzed in the cultured HMEC of four donors (B, D, E, and F). Immunoblot analysis identified the expression of the 97kD protein in all individuals examined, the level of which varied by 3-fold (Fig. 9A). TCDD-induction resulted in the down-regulation of the cytosolic receptor (approximately 3-fold), which is mediated by nuclear translocation followed by proteolytic turnover (56). The level of receptor expression was many times higher in the cultured HMEC, as compared to MDA-MB-231 cell line, the human mammary carcinoma cell line shown to express the highest level of AhR expression among several mammary cell lines examined (57).

The expression of Arnt was also much higher in the cultured HMEC, relative to the MCF-7 and MDA-MB-231 human mammary carcinoma cell lines (Fig. 9B). The level of Arnt expression varied by only 1.5-fold among the four donors.

DISCUSSION:

This study has characterized functional CYP1B1 and CYP1A1 expression in early passage HMEC isolated from tissues procured from eight women undergoing reduction mammoplasty surgeries. The roles of CYP1B1 and CYP1A1 in PAH metabolism have been quantitatively characterized under conditions which reflect the basal condition of the cultured HMEC. Thus, the potential for PAH-mediated induction of CYP1B1 and CYP1A1 has been eliminated by maintaining short PAH reaction periods in the intact cellular assay and by directly assaying microsomal metabolism. Cytochrome P450 induction has been studied utilizing a non-metabolizable Ah-receptor agonist, TCDD, which substantially induced both isoforms following a 24 h exposure. Through the immunoquantitation of each form, we can account for PAH metabolism in terms of the expressed level of these two P450 cytochromes under both basal and TCDD-induced conditions. Previous studies of PAH metabolism in cultured HMEC have employed prolonged exposures of the hydrocarbons to the cells (24-42 hours), thereby, combining P450-induction with P450-mediated bioactivation events (15, 41-42). In addition, these studies

have not addressed which specific P450 isozyme(s) are responsible for PAH metabolism and bioactivation.

In this study, the HMEC have been cultured under conditions similar to those applied in the previous studies of HMEC-mediated PAH metabolism which, as we have confirmed, provides a mixture of luminal and basal epithelial cell populations. Under these culture conditions, the proportion of basal HMEC progressively increase with increased passage and time in culture relative to the luminal subtype (45). In parallel with a predicted increase in the proportion of basal cells (45), we observed a marked decrease in TCDD-induced DMBA metabolism and CYP1A1 microsomal protein expression following day 6 of secondary culture, and continuing into passage 2 cultures. This change would be consistent with the predominant expression of CYP1A1 in the luminal cell population, while CYP1B1 continued to be expressed in the basal epithelia. We have also observe increased variability in CYP1A1 expression, potentially arising from differing proportions of the luminal cells among the individual donors. This is being addressed in ongoing studies. Interestingly, by far the lowest CYP1A1 expression was observed in very slow growing cultures from donors A and C.

The present study demonstrates that CYP1B1 is expressed constitutively, at quantifiable levels, in cultured HMEC from seven of the women. In contrast, constitutive CYP1A1 is expressed at extremely low levels (approaching the lower limit of detectability), although both P450 cytochromes are expressed at comparable levels (pmol P450/mg microsomal protein) in the HMEC following TCDD exposure. Northern hybridization and rtPCR analyses demonstrate that mRNA expression parallels levels of the respective microsomal protein in these cells, establishing that the cytochrome P450 expression is transcriptionally regulated. The levels of immunodetectable constitutive and TCDD-inducible CYP1B1 expression each varied by approximately 2.5-fold (excluding donor A), while induced levels of CYP1A1 varied by as much as 21-fold among the eight individuals. At present we do not know the extent to which this reflects inter-donor differences in the proportions of basal and luminal epithelial cells or differences in CYP1A1 expression.

Although DMBA is a highly discriminating substrate for the functional measurement of rodent CYP1A1 and CYP1B1 (44), elucidating the individual contributions of these forms to DMBA metabolism has been difficult in human cells. While rodent CYP1B1 and CYP1A1 demonstrate highly distinct regioselective profiles of DMBA metabolite production, the corresponding profiles are less distinct for the human P450 cytochromes. For example, in rodents CYP1A1-mediated metabolism is characterized by the preferential hydroxylation at the 7-methyl substituent of DMBA and the formation of the 8,9-DMBA-dihydrodiol, whereas CYP1B1 selectively metabolizes DMBA to produce high levels of the 10,11- and 3,4-dihydrodiol metabolites. However, by utilizing recombinant human CYP1B1 and CYP1A1 we have shown

that human CYP1A1-mediated metabolism is characterized by the preferential formation of the 8,9-DMBA-dihydrodiol with proportionally less 5,6-dihydrodiol and 7-hydroxy-DMBA than for rodent CYP1A1. There is also clear production of the 3,4-dihydrodiol which is absent with the rodent ortholog. Human CYP1B1-directed metabolism yielded relatively high proportions of the 5,6- and 10,11-DMBA-dihydrodiol metabolites, relative to human CYP1A1, but unlike rodent CYP1B1, showed a similar proportion of the 3,4-dihydrodiol (5 percent). Human CYP1A1 is 6-fold more efficient in the metabolism of DMBA, as compared to CYP1B1, a much larger difference than observed in the rodent forms.

Secondary HMEC cultures demonstrated low levels of constitutive microsomal DMBA metabolism which was mediated by CYP1B1, consistent with the immunodetectable basal expression of this isoform. The regioselective profile of metabolite distribution generated from these HMEC was consistent with that produced by human recombinant CYP1B1 and this has been confirmed by the selective inhibitory effect of anti-CYP1B1 on DMBA metabolism, as compared to the lack of inhibition with anti-CYP1A1 antibodies. Small, but significant, differences in the regioselective profile of metabolite formation were observed between the HMEC and the recombinantly expressed protein (HMEC demonstrate a lower 5,6-/10,11- dihydrodiol ratio). This may be due to differences in the configuration of CYP1B1 in HMEC and in lymphoblast endoplasmic reticulum. We have recently measured product distribution for DMBA metabolism by recombinant human CYP1B1 expressed in V79 cells which more closely matches the HMEC product ratios (data not shown). Certainly, for each of these sources, the mobility of the CYP1B1 proteins in SDS-PAGE is indistinguishable. Donor E, one of a group of three individuals demonstrating low levels of basal CYP1A1 immunodetectable expression, yielded a profile of regioselective basal metabolism reflecting approximately equal contributions from both CYP1A1 and CYP1B1. Although the level of basal CYP1A1 expression in donor E is approximately 10 times lower than basal CYP1B1, the contribution of CYP1A1 to DMBA metabolism is consistent with the 6-fold higher turnover of the isoform. This provides a critical confirmation of the immunodetection of constitutive CYP1A1 in these cultures. The consistent finding of CYP1A1 in cells from these donors, and not others cultured in the same media, indicates that this is not due to an inducer in the media but, rather, results from a selective endogenous activation of CYP1A1 with these donors.

Exposure of the cultured HMEC to TCDD increased the overall rate of DMBA metabolism by 30- to 40-fold. Although TCDD-induced CYP1B1 and CYP1A1 were expressed at equivalent levels, the induced metabolism was predominantly mediated by CYP1A1. The profile of metabolite regiodistribution paralleled that of recombinant human CYP1A1. Since CYP1A1 is approximately 6-fold more efficient than CYP1B1 in metabolizing DMBA, we would predict, at equimolar levels of expression, CYP1A1 and CYP1B1 to mediate 86% and 14% of induced

DMBA metabolism, respectively. Indeed, antibody inhibition analysis confirmed this activity distribution, within the sensitivity of the assay. We have shown that anti-CYP1A1 substantially inhibited metabolic activity, while yielding a regioselective profile of residual metabolism indicative of predominantly CYP1B1-mediated activity. Furthermore, much of the residual activity was removed by a further addition of anti-CYP1B1 antibodies.

We have estimated the turnover number for DMBA metabolism with the exceptionally low levels of the individual forms of cytochrome P450 calculated in this study. This involves comparison of expression levels with the proportion of the metabolism attributable to the individual form based on antibody inhibition. Constitutive activities from donors D and E indicate CYP1B1 turnover of 6 h^{-1} in excellent agreement with the turnover of recombinant human CYP1B1 in lymphoblast microsomes (4 h^{-1}). CYP1A1 turnovers were estimated at 90 h^{-1} in constitutive microsomes from donor E and at 21 h^{-1} in TCDD-induced microsomes. Again, these rates compare remarkably well with the activity of recombinant human CYP1A1 in lymphoblast microsomes (17 h^{-1}).

Cellular DMBA metabolism was fully consistent with the microsomal activity. Both assays demonstrate TCDD-inducible metabolic activity and the regioselective distribution of metabolites was consistent between the two methods of analysis. In cells, approximately 60% of dihydrodiols were released by β-glucuronidase, implicating their conjugation as glucuronides. This agrees with previous measurements of these conjugation ratios (42). The cellular and microsomal assays yielded an equivalent overall rate of metabolic activity, based upon a recovery of 1 mg of microsomal protein/10^7 cells. Interestingly, we have found as much as 40% of the functional activity is in cell fractions which contain nuclear and perinuclear membranes. The cellular analysis of basal metabolism did not demonstrate measurable activity above non-enzymatic background of DMBA oxidation. Based on the microsomal assays, we would not expect to detect DMBA metabolism from these cellular assays, which typically contained only 10^5 cells.

AhR levels in the cultured HMEC were at least 5-fold elevated relative to the highest level observed in an established human mammary cell line (MDA-MB-231 cells). TCDD-mediated down-regulation of the cytosolic receptor level clearly showed that the cells in culture were expressing a functional receptor, most of which translocated to the nucleus upon binding the ligand and is degraded concomitant with nuclear activation (i.e. enhanced transcription of CYP1A1 and CYP1B1). Since the levels are over 50 times higher than in tumor cell lines, such as MCF-7 where TCDD is similarly effective in inducing these genes, apparently the AhR is not a limiting factor mediating cytochrome P450 expression in HMEC. This raises major questions regarding the functional significance of such high levels of AhR expression in these cells. Similarly, Arnt, the nuclear partner of the AhR, was elevated in the cultured HMEC, as compared to the human mammary cell lines. It appears that when the AhR translocates to the nucleus there is sufficient

ARNT for heterodimerization of these proteins. Since there is unlikely to be an increased requirement for binding to gene enhancer binding elements, this high level may function to complex and regulate additional nuclear factors.

This study has confirmed that the mammary epithelia of certain individuals constitutively express levels of CYP1A1 which are extremely low but, nevertheless, are functionally significant in PAH activation. Although the level of immunodetectable constitutive CYP1A1 expression is 7- to 70-fold lower than the level of CYP1B1 in these individuals, and is only barely detectable by rtPCR methodologies, the CYP1A1 expression has been demonstrated by the correlative expression of protein and mRNA with metabolic activity analyses. This activity suggests that any individual expressing CYP1A1 in the mammary gland in this way is at an increased risk for PAH-bioactivation, bearing in mind that the level of PAH exposure/bioaccumulation is maintained well below inducing levels due to constitutively active P450's, such as CYP1B1.

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Table 1. **Summary of Experiments Completed on HMEC Obtained from Eight Individual Donors.**

DONOR	AGE	IMMUNOBLOT ANALYSES				DMBA METABOLISM	rtPCR	NORTHERN HYBRIDIZATION
		CYP1B1	CYP1B1	AHR	ARNT			
A	17	+	+				+	
B	19	+	+	+	+	+	+	
C	20	+	+			+		
D	22	+	+	+	+	+	+	+
E	22	+	+	+	+	+		+
F	35	+	+	+	+			
G	40	+	+					
H	49	+	+					

Table 2. Analysis of Cellular Fractionation of TCDD-Induced DMBA Dihydrodiol Metabolite Formation in Day 6 HMEC.

PREPARATION[a]	DMBA Dihydrodiols 5,6-	8,9-	10,11-	3,4-	TOTAL
Intact Cells	1.9 (16)[c]	7.9 (67)	1.8 (15)	0.2 (2)	**11.8**[b]
Microsomes	18.5 (14)	97.1 (73)	10.5 (8)	7.3 (5)	**133.5**[d]
Nuclear/ Mitochondrial Pellet	22.1 (25)	45.0 (53)	16.0 (19)	2.6 (3)	**85.7**[d]

[a] HMEC were obtained from Donor D.
[b] pmol/10^6 cells/h.
[c] Percent distribution of metabolites.
[d] pmol/mg/h.

TABLE 3. Microsomal DMBA Metabolism in Constitutive and TCDD-Induced HMEC.

SAMPLE	Dihydrodiols 5,6-	8,9-	10,11-	3,4-	7-OHMMBA	Phenols A	B	Total DMBA Metabolism (pmol/mg/hr)
CONSTITUTIVE								
Donor D	0.3	0.6[a]		0.2	0	1.6	0.6	**3.3**
	(9)[b]	(18)		(6)		(48)	(18)	
Donor E	1.1	2.4	0.4	0.4	0	4.2	0.6	**9.1**
	(12)	(27)	(4)	(4)		(46)	(7)	
TCDD-INDUCED								
Donor D	26	147	7	9	35	67	18	**309**
	(8)	(48)	(2)	(3)	(11)	(22)	(6)	
Recombinant Human Cytochromes P450								
CYP1B1[c]	50	33	27	17	4	154	2	**287**
	(17)	(12)	(10)	(6)	(1)	(54)	(<1)	
CYP1A1[d]	189	870	73	80	10	450	45	**1717**
	(11)	(51)	(4)	(5)	(<1)	(26)	(3)	

[a] DMBA 8,9 + 10,11-dihydrodiol, peaks were poorly resolved at this low level of activity.

[b] Percent metabolite distribution.

[c] Recombinant human CYP1B1 expressed in lymphoblast microsomes (Gentest Corp.), 74-pmol/mg protein.

[d] Recombinant human CYP1A1 expressed in lymphoblast microsomes (Gentest Corp.), 104-pmol/mg protein.

TABLE 4. Antibody Inhibition of Microsomal DMBA Dihydrodiol Formation in HMEC.

Treatment / IgG Addition	Dihydrodiols				Total Dihydrodiol Formation (pmol/mg/hr)	% Inhibition
	5,6-	8,9-	10,11-	3,4-		
Constitutive						
(+) pre-immune[a]	0.29	0.34[d]		0.19	**0.82**	**0**
(+) anti-1A1[b]	0.29	0.35		0.12	**0.76**	**7**
(+) anti-1B1[c]	0.07	0.05		0.01	**0.13**	**84**
Δ anti-1B1	0.22	0.29		0.18	**0.69**	
TCDD-Induced						
(+) pre-immune[a]	17	68	12	7	**104**	**0**
(+) anti-1A1[b]	6	10	4	2	**22**	**79**
(+) anti-1B1[c]	10	55	8	5	**78**	**25**
(+) anti-1A1+anti-1B1[b,c]	2	5	2	1	**10**	**90**
Δ anti-1A1	11	58	8	5	**82**	

[a]Metabolism demonstrated by donor D.
[b]10mg IgG/mg microsomal protein.
[c] 5mg IgG/mg microsomal protein.

[d] 8,9-+10,11- DMBA dihydrodiols, peaks were poorly resolved at this low level of activity.

TABLE 5. Antibody Inhibition of Constitutive DMBA Dihydrodiol Formation in HMEC of Donors D and E.

Treatment / IgG Addition	Total Dihydrodiol Formation (pmol/mg/hr)	% Inhibition
Donor D		
(+) pre-immune	**0.82**	**0**
(+) anti-1A1[a]	**0.76**	**7**
(+) anti-1B1[b]	**0.13**	**84**
Donor E		
(+) pre-immune	**2.31**	**0**
(+) anti-1A1[a]	**1.48**	**36**
(+) anti-1B1[b]	**1.52**	**34**
(+) anti-1A1+anti-1B1[a,b]	**1.19**[c]	**48**

[a]10mg IgG/mg microsomal protein.
[b] 5mg IgG/mg microsomal protein.
[c]Inhibition analysis consistently yields a contaminating peak with a retention time corresponding to that of the 10,11-dihydrodiol of DMBA.

FIGURE LEGENDS:

Figure 1. Primary HMEC organoid preparations cultured in MEGM media exhibiting regions of luminal (A), basal (B), and mixed luminal and basal (C) epithelial cell proliferation.

Figure 2. Total cellular DMBA metabolism of TCDD-induced HMEC at day 4, 6, and 13 of culture. Secondary HMEC cultures of donor D were maintained in MEGM media and were treated with 10nM TCDD for 24 h prior to analysis as described in Materials and Methods.

Figure 3. Immunoblot analysis of CYP1B1 (A., 1 min exposure) and CYP1B1 (B., 2 min exposure; C., 10 min exposure) expression in secondary HMEC cultures of 6 individual donors. Constitutive (C) and TCDD-induced T (10nM for 24 h) microsomal proteins were analyzed by SDS-PAGE and the immunoreactive proteins were visualized using the ECL method as described in Materials and Methods. Microsomal protein loadings were as indicated. Recombinant human CYP1B1 (74-pmol/mg) and CYP1A1 (104-pmol/mg) expressed in lymphocyte microsomes (Gentest Corp.) were utilized as standards for the quantitation of CYP expression.

Figure 4. Quantitation of CYP1B1 (A., TCDD-induced; B., constitutive) and CYP1A1 (C., TCDD-induced; D., constitutive) microsomal protein expression (pmol/mg) in secondary HMEC cultures of seven donors. CYP expression was quantitated from the standard curves shown in Figure 3 by soft laser scanning laser densitometry.

Figure 5. The correlative expression of the level of TCDD-induced (10nM, 24 h) CYP1B1 and CYP1A1 microsomal protein (pmol/mg) in secondary HMEC cultures of seven donors.

Figure 6. Immunoblot analysis of CYP1B1 (A, 1 min exposure) and CYP1B1 (B, 2 min exposure; C, 10 min exposure) expression in passage 1 and passage 2 (*) HMEC. Immunoblot analysis was completed as described in Figure 3.

Figure 7. rtPCR analysis of CYP1B1 and CYP1A1 mRNA expression in HMEC cultures of three donors. Total RNA was isolated from constitutive (C) and TCDD-induced (T, 10nM for 6 h) day 6 secondary cultures using TRIzol methodologies. cDNA was synthesized and PCR amplifications were completed as described in Materials and Methods. PCR reaction products were analyzed by agarose gel (1.6 % agarose) chromatography and visualized by staining in ethidium bromide. TCDD-induced MCF-7 cDNA was utilized as a positive control, while the (-) control was devoid of cDNA. MCF-7 cDNA standardization has shown that a 10-fold difference in message corresponds to a 3-fold difference in ethidium bromide signal intensity (unpublished observation).

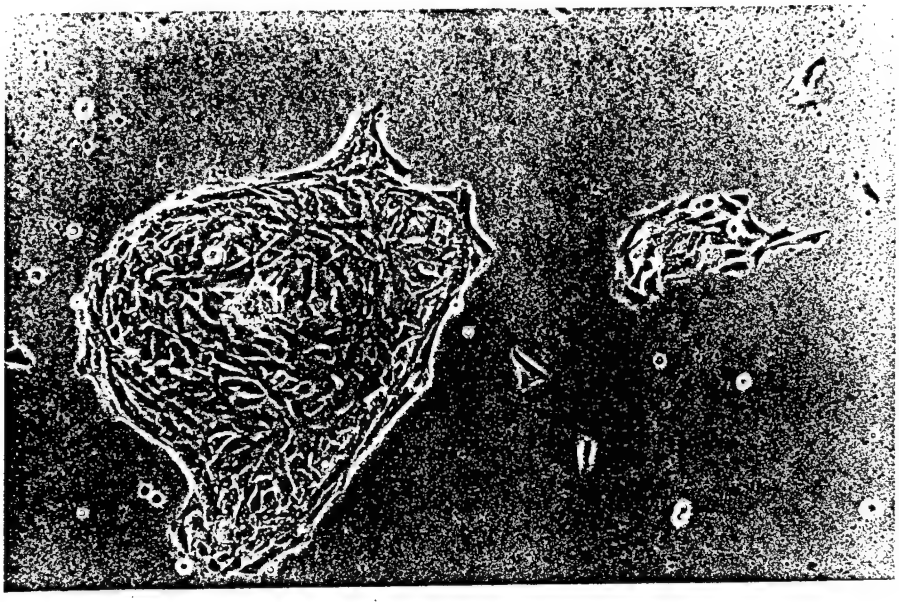
Figure 8. Analysis of CYP1B1 mRNA expression in cultured HMEC. RNA was isolated from constitutive (C) and TCDD-induced (10 nM for 6 h) secondary HMEC cultures as described in Materials and Methods. Poly(A)$^+$ RNA (10 μg/lane) was probed for CYP1B1 using a rat CYP1B1 cDNA probe and loadings were normalized to β-actin. Note: Donor D samples were visualized by autoradiography film with exposure times of 60 h (CYP1B1) and 18 h β-actin. The remaining samples were visualized by phosphoimager analysis with exposure times of 18 h (CYP1B1) and 3 h (β-actin).

Figure 9. Immunoblot analysis of AhR (A, constitutive (C) and TCDD-induced (T, 10nM for 24 h) and Arnt (B, constitutive) expression in day 6 secondary

HMEC cultures of four individual donors. Total proteins were isolated by TRIzol procedures and were analyzed by SDS-PAGE. Immunoreactive proteins were visualized by the ECL method as described in Materials and Methods. MDA-MB-231 and MCF-7 protein fractions were utilized as reference standards for comparison with expression levels in immortalized human mammary cell lines. Protein loadings were 20μg / lane and membranes were exposed for 5 minutes.

Figure 1

A.



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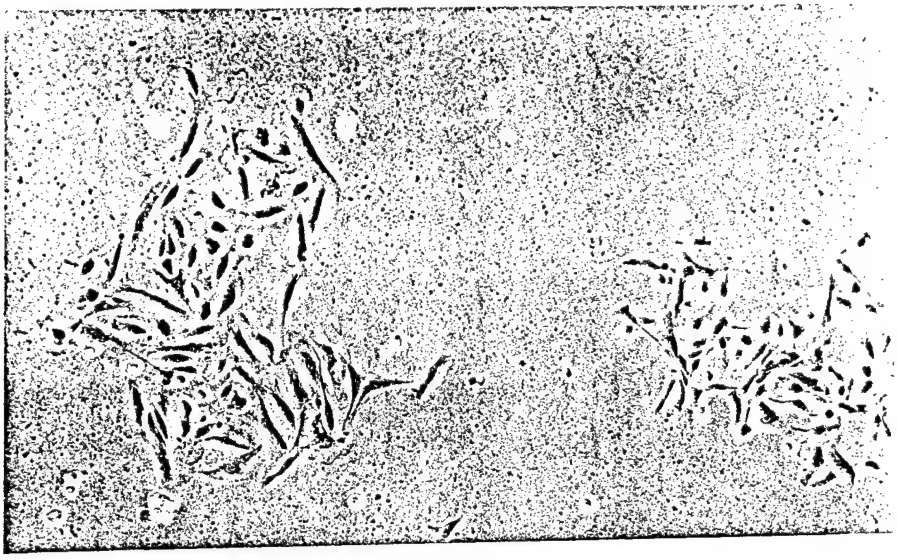


C.

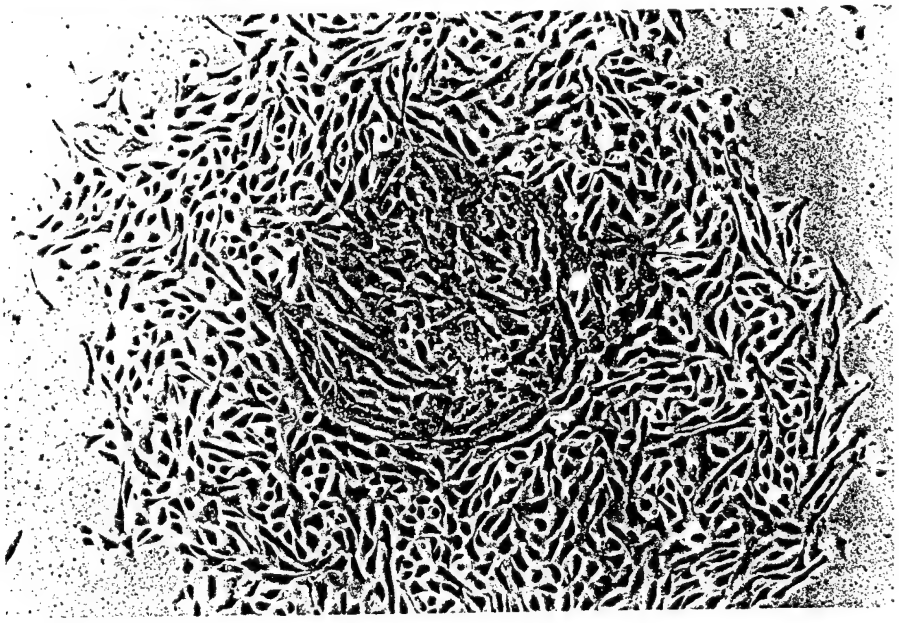


Figure 2. Primary HMEC organoid preparations cultured in MEGM media exhibiting regions of luminal (A), basal (B), and mixed luminal and basal epithelial cell proliferation.

Figure 2

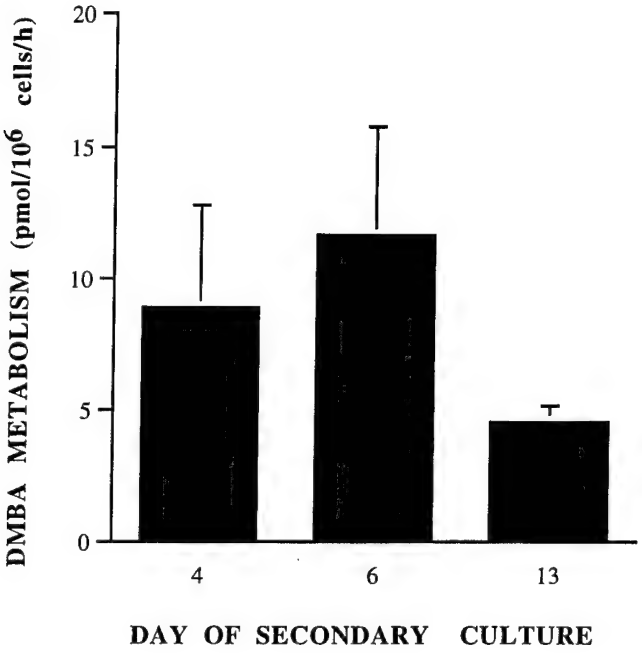

DMBA METABOLISM (pmol/10^6 cells/h)
20
15
10
5
0
4
6
13
DAY OF SECONDARY CULTURE

Figure 3

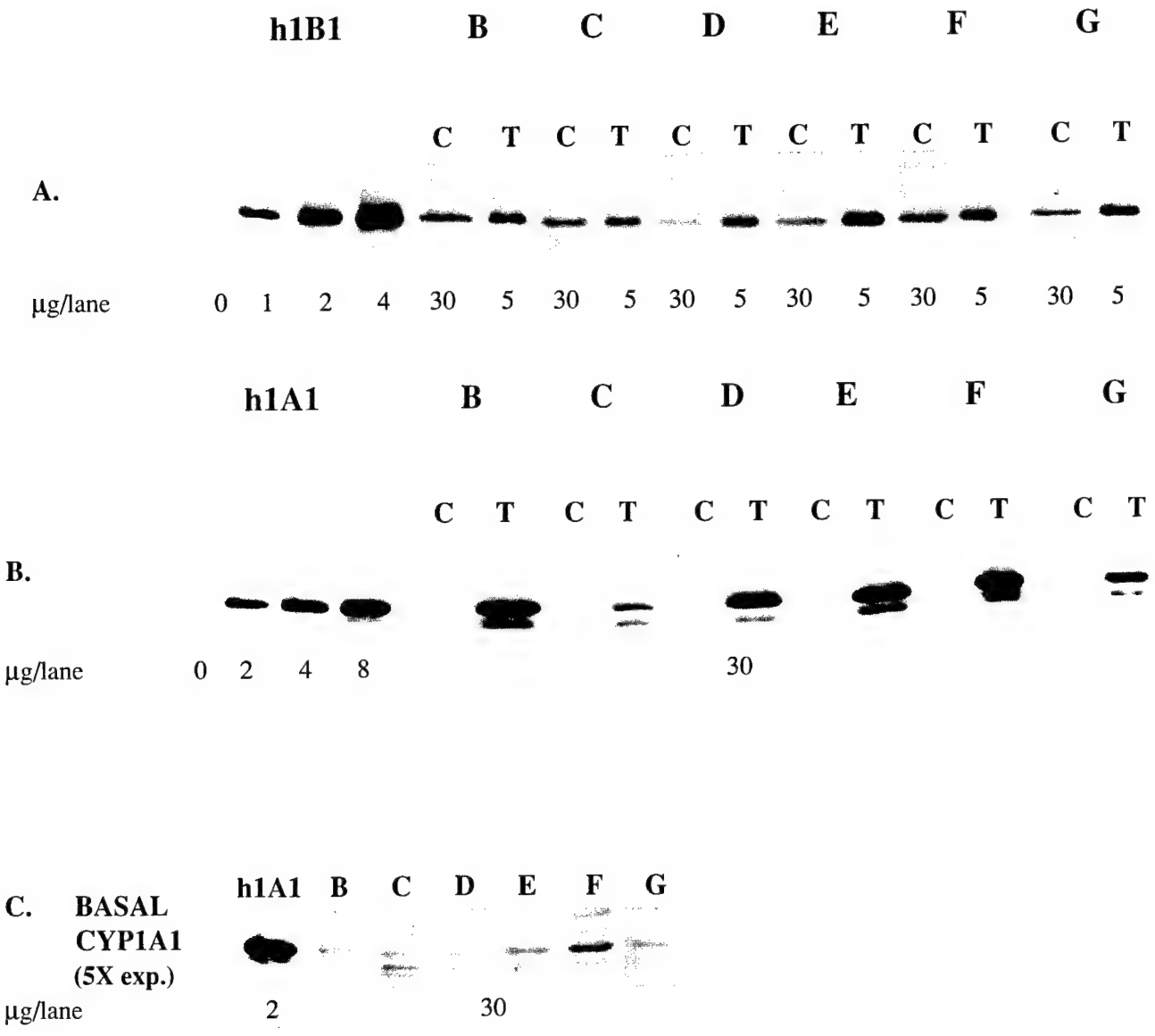

h1B1
B
C
D
E
F
G
C T C T C T C T C T C T C T
A.
µg/lane 0 1 2 4 30 5 30 5 30 5 30 5 30 5 30 5
h1A1
B
C
D
E
F
G
C T C T C T C T C T C T C T
B.
µg/lane 0 2 4 8 30
C. BASAL CYP1A1 (5X exp.)
h1A1 B C D E F G
µg/lane 2 30

Figure 4

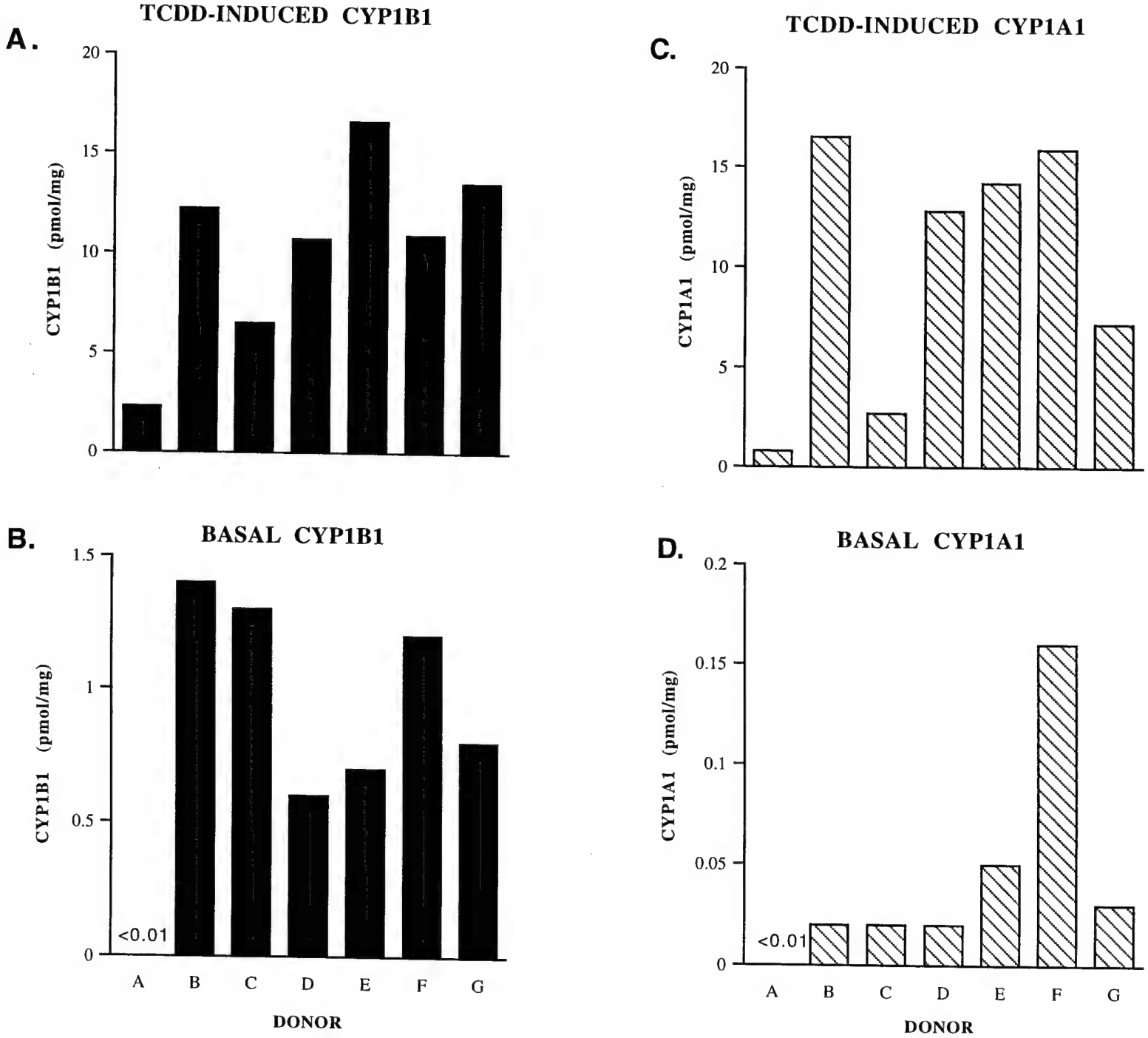
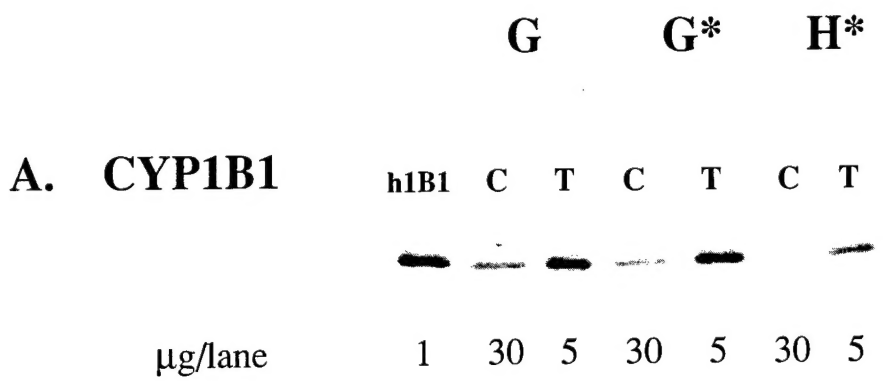
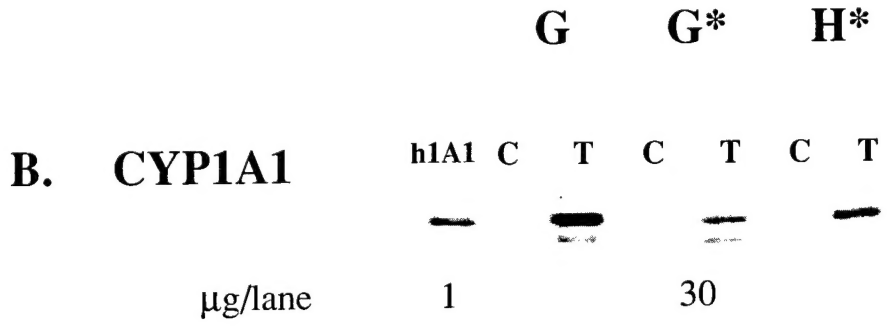

A.
TCDD-INDUCED CYP1B1
CYP1B1 (pmol/mg)
20
15
10
5
0
B.
BASAL CYP1B1
CYP1B1 (pmol/mg)
1.5
1
0.5
0
<0.01
A
B
C
D
E
F
G
DONOR
C.
TCDD-INDUCED CYP1A1
CYP1A1 (pmol/mg)
20
15
10
5
0
D.
BASAL CYP1A1
CYP1A1 (pmol/mg)
0.2
0.15
0.1
0.05
0
<0.01
A
B
C
D
E
F
G
DONOR

Figure 5 not available
for this publication
Will be added upon publication

Figure 6


G
G*
H*
A. CYP1B1
h1B1 C T C T C T
µg/lane 1 30 5 30 5 30 5


G
G*
H*
B. CYP1A1
h1A1 C T C T C T
µg/lane 1 30

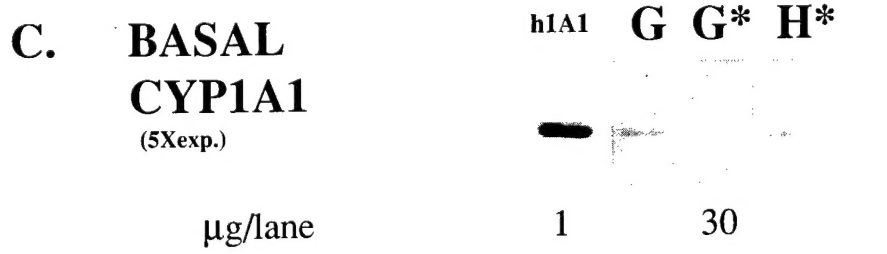

C. BASAL CYP1A1
(5Xexp.)
h1A1 G G* H*
µg/lane 1 30

Figure 7

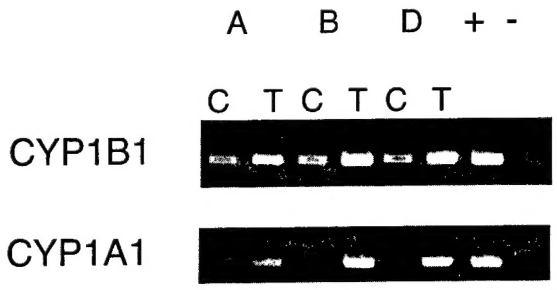


Figure 8

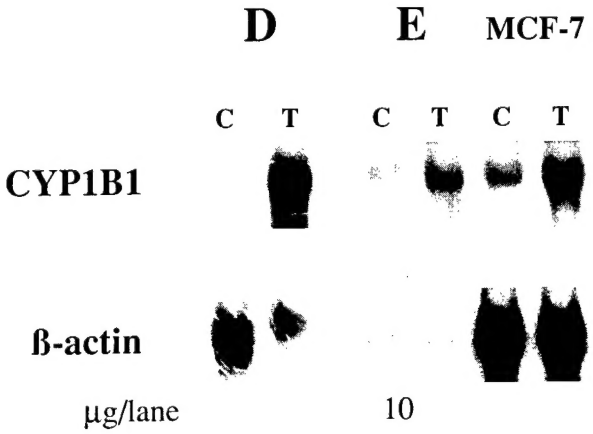


Figure 9